volume 23, no. 5

sept.-oct. 1958

BIOCHEMISTRY

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227 WEST 17TH STREET, NEW YORK 11, N. Y.

volume 23, no. 5

Sept.-Oct. 1958

BIOCHEMISTRY

translated from Russian

BIOKHIMIIA

A publication of the Academy of Sciences of the USSR

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CONSULTANTS BUREAU, INC.

227 W. 17th St., NEW YORK 11, N. Y.

Year and issue of first translation:
volume 21, no. 1, jan.-feb. 1956

Annual Subscription	*$20.00*
Single Issue	*10.00*

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The National Institutes of Health of the Public Health Service has supported in part the cost of publishing BIOCHEMISTRY through the purchase of copies for distribution to American medical libraries.

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A PROBLEM OF GREAT IMPORTANCE

In May of the current year the Central Committee of the Communist Party of the Soviet Union through its Plenum considered the speech of the first secretary of the Central Committee, Comrade N. S. Krushchev, "On Hastening the Development of the Chemical Industry and Especially the Production of Synthetic Materials and Manufactured Materials from Them to Satisfy the Needs of the People and the Requirements of the National Economy" and adopted a resolution on this speech.

The decision of the May Plenum of the Central Committee indicates a new era in the development of chemistry and will have great consequences for all branches of national economy and for all our lives.

It is generally known that the Soviet chemical industry has made great progress, especially in recent years. The volume of its production in 1957 was five times greater than in 1940. In tonnage our chemical industry has reached second place in the world, but is still surpassed by that of the United States of America.

The pioneer work of Russian and Soviet scientists has played an important part in the development of a number of important branches of chemical industry. The fundamental work of D. I. Mendeleev, the theory of chemical structure proposed by the great Russian chemist, A. M. Butlerov, the deep investigations of the Russian and Soviet schools of chemists have provided a firm foundation for the development of the present chemical industry.

Especially characteristic of the development of the chemical industry in the last decade has been the rapid growth in the production of high molecular weight materials, plastics and resins, artificial fibers, and the raw materials for them. As early as 1955 the production of these materials in capitalistic countries passed 5 million tons and it still rises. The production of plastics and artificial fibers has risen especially fast. The world production of plastics increased eight times from 1945 to 1957, from 500,000 tons to 4,100,000 tons per year.

The chemistry of high molecular weight compounds has opened new paths for supplying various materials with previously desired properties.

Science has opened wide paths for human advantage. It will always supply new technological principles and new methods of production. This is very important for the development of synthetic chemistry. Many fields of chemical industry are created by principles which are best carried out in the living body for the formation of various substances.

A clear example of the economy of biosynthesis compared to the industrial synthesis is the biological fixation of nitrogen by azotobacter and the nodule bacteria of leguminous plants compared to the industrial synthesis of ammonia. While the first occurs under ordinary conditions of temperature and pressure, the second requires high pressures and high temperatures which greatly increase the cost.

The differences are chiefly due to the fact that the biosynthesis is regulated by the proper enzymes, that is, by those substances which work in living organisms and hasten the reactions millions of times. In industrial syntheses the catalysts used are much less perfect and do not permit the same gentle conditions which occur in living bodies.

Finally, the specificity of the chemical processes in the living cell is determined not only by the nature of the catalyst, but also by the very complex structures and the molecular relations under the concrete conditions of life processes which are eventually determined by the specific dynamic nature of the living body. Here we must also remember that not all biological syntheses are based on the monotonous principles that are so charac-

teristic for the chemical synthesis of high molecular weight compounds. In biosynthesis, besides the monotonous cases, many important compounds are formed by structural bonds between complex molecules of different types.

We could give many examples which would confirm the importance of studying the rules of biological synthesis for use in chemical industry in the production of new materials and supplying new technological principles.

It is also necessary to consider that biologically produced polymers lie at the basis of living functions. Therefore the development of the chemistry of high molecular weight compounds in its turn may have great value for the understanding of intimate life processes. There is no doubt that realization of the plans of the May Plenum will involve working out problems in modern biochemistry. The study of enzymes, the laws which govern their actions, questions of structure and artificial synthesis of proteins, nucleic acids, nucleoproteins, hormones, the chemical nature of viruses and the laws of their reproduction, study of high-polymer carbohydrates, immune bodies, and many other natural compounds, all these are of importance not only for an understanding of living phenomena, but also for the development of many fields of industry which utilize raw materials produced by plants and animals.

We can give several examples of the use of biological polymers in industry. The progress of biochemistry has shown its great value in many fields of the enzyme industry. An understanding of the properties and behavior of enzymes allows us to set up the problem of improving their action in the treatment of technological raw materials. Soviet biochemists have made great progress in this direction in the last 20-25 years. They have worked out and introduced a more rational method for technical processes in many fields of the food industry, bakeries, alcohol factories, breweries, starch-molasses plants, wineries, tea and tobacco factories, etc. The problems relate chiefly to improving the action of the enzymes which are contained in the raw materials.

Much greater possibilities have been shown recently in the technical production of enzyme preparations in concentrated or pure form from cheap starting materials. The advantage of using enzyme preparations is so great that it has caused a rapid growth in the demand for them and the development of special industries for their production. The greatest development of these industries has occurred in the United States, England, and Japan. In the USA there are more than 10 specialized firms for the production of enzymes from fungi and bacteria. In Japan they produce up to 7000 tons of preparations of one bacterial amylase, with which they have reconstructed the technology in a number of fields in food and light industry.

The use of enzyme preparations permits intensification in production processes, lowers the cost of expensive foods, raises the quality of production, and supplies new substances of technical, nutritional, and medical value. Thus, according to the experiments of the All-Union Institute of the Baking Industry (Moscow) the introduction of yeast amylase into white flour greatly increases the quality of the bread. In the production of some kinds of bread there is not only an improvement in quality, but there is no longer any need to add malt which had required the use of a large amount of grain. According to the All-Union Institute of the Baking Industry the elimination of malt by the use of enzyme preparations in a bakery producing 60 tons of bread a day results in a saving of about 700,000 rubles per year.

In the alcohol industry enzyme preparations can also eliminate malt which would permit an economy of tens of thousands of tons of treated grain. It is interesting that the yield of alcohol would also be raised.

In food industries, enzyme preparations can be widely used to combat turbidity, to stabilize foods, and to lengthen the period of keeping. In the starch – molasses industry enzymes can give a new type of production with definite nutritive properties. Enzymes can be used widely in the production of fructose and berry juices, in the wine industry, etc. The use of enzyme preparations will shorten the production of champagne and will permit a marked improvement in quality and an increase in output of champagne.

Aside from the food industries, enzyme preparations are needed to improve the technical processes of the textile, silk, and tanning industries.

Many causative agents for agricultural plant diseases quickly become adapted to chemical poisons and effective measures against them become difficult. An entirely new prospect has been discovered here in the virological method of attack.

Recently yeast and bacterial enzyme preparations have found wide use in medical practice, replacing less active or pure preparations produced by animals (pancreas, gastric juice). They are used to improve

nutrition in many diseases of the gastrointestinal tract, and also for the treatment of wounds, ulcers, suppurations, etc., in order to remove the dead tissue quickly and harmlessly, and promote more rapid healing.

Finally, new prospects have been open in blood substitutes. England now produces many tons of polymers which can substitute for blood.

These examples show that in the realization of the plans for the development of chemical industry the biological front cannot be neglected, especially the need for the development of biochemistry.

We speak not only of strengthening particular biochemical establishments, but also the wide application of related fields of knowledge for working out biochemical problems. It is important that biochemical problems evoke interest among physicists and chemists so that biochemical questions will be treated not only by biochemists, but by all scientists who will apply their own modern experimental techniques.

The most important factors for assuring a progressive development of biochemistry in our country are the following:

a) strengthening the existing basis of biochemical establishments and organizing new ones;

b) attracting physicists and chemists for coordinated work in the field of biochemistry;

c) wide introduction of biochemical methods and biochemical approaches to the solution of physiological and general biological problems;

d) development of apparatus manufacture and particularly the production of high speed centrifuges, new optical and electron-optical apparatus, and organization of the production of biochemical preparations, particularly compounds labeled in various positions;

e) initiation of periodicals which review different phases of current biochemistry, such as the annuals now issued in other countries;

f) taking the necessary measures to hasten publication of experimental work, widening the education of biochemists, and strengthening the physicochemical training of young specialists;

g) introduction of biochemistry courses in agricultural, technical, and other educational institutions where specialists are prepared in fields concerned with treatment or preservation of materials of plant or animal origin.

The development of science proceeds on the basis of new facts and generalizations. However, facts may be used separately from generalizations depending on the views and methodology of the scientist. Timely, original, materialistic generalizations of experimental investigations have great value for the progress of science itself. However, facts are the cornerstone, proved true by scrupulous and repeated tests; facts which remain true after many tests by direct and indirect methods have material strength; facts produce wide repercussions in many laboratories and often cast new lights, and in many cases refute established and customary postulates of science.

Our problem is to use all the possibilities which we have to develop biochemistry so that it will reach the level of those problems and requirements which come from the May Plenum of the Central Committee.

PHYSICOCHEMICAL MECHANISM OF PHOTOREACTIVATION OF BACTERIA WHICH HAVE BEEN SUBJECTED TO ULTRAVIOLET IRRADIATION

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The photoreactivation of bacteria which have been exposed to inactivating doses of ultraviolet irradiation was first reported in 1949 [1]; the present paper presents the results of a research designed to elucidate the chemical processes associated with the resuscitation of such bacteria. This phenomenon is of wide incidence, as it has been shown to occur among some of the lower forms of animal life [2], as well as among bacteria.

Ultraviolet light, particularly of a wave length of 2537 A, which is strongly absorbed by nucleic acids, inhibits the growth of bacteria. This effect may be abolished by subsequent exposure of the bacteria to light of longer wave length, from the visible part of the spectrum [3].

Apart from their growth-inhibitory effect, ultraviolet irradiation has a mutagenic effect, which can also be abolished by subsequent photoreactivation. The mutagenic effect of ultraviolet irradiation, and its subsequent abolition by visible light, can be demonstrated repeatedly, by exposure to ultraviolet and visible light, alternately [4].

The process of inhibition of bacterial growth by ultraviolet irradiation is associated with a fall in the desoxyribonucleic acid content per mg of bacterial mass [5], with a concomitant rise in the acid-soluble fraction, i.e., in purine and pyrimidine compounds, in organic, acid-soluble phosphate, and in ribose and desoxyribose [6]. This fall in the nucleic acid content is regarded as resulting from suppression of synthesis of nucleic acids by the irradiated organisms [5]. The accumulation of low molecular products is similarly regarded as being due to inhibition of integration of low molecular weight precursors into the desoxyribonucleic acid macromolecules [6, 8].

The photoreactivation of bacteria on exposure to visible light is evidently connected with restoration of the initial desoxyribonucleic acid content, and hence with normalization of the processes of its synthesis [5]. Photoreactivation of bacteria which have been inactivated by exposure to ultraviolet irradiation may be total, if visible light is allowed to act immediately after ultraviolet irradiation. Inactivation of the organisms becomes irreversible if illumination is delayed for 2-3 hours; this effect has not yet been explained [7].

It is reasonable to assume that ultraviolet irradiation causes depolymerization of nucleic acids, with accumulation of the resulting fragments. Photoreactivation by light of longer wave length, having a lower quantum energy, could lead to reconstitution of these fragments, to restore the initial desoxyribonucleic acid content of the bacteria. The larger DNA fragments might for some time be sufficiently close to each other to permit of their recombination [9], using, for example, the energy derived from photoreactivation.

According to this concept of the mechanism of the action of ultraviolet and visible light, the absence of photoreactivation observed when the irradiated bacteria are kept in the dark for a certain length of time, in nutrient medium or in buffer solutions, could be ascribed to the extrusion into the medium of nucleic acid fragments. The continuing depolymerization of desoxyribonucleic acids would lead to accumulation of low molecular weight fragments, which would carry functional groups. The density of such functional groups per unit surface

of bacterial substance, and hence the density of the charge, would increase as long as the fragments of depolymerized DNA remained with the irradiated bacterial cells. If diffusion of such fragments took place into the medium when the irradiated cells were kept for a certain time in the dark, the density of charge per unit area of bacterial surface should diminish. In addition, it should be possible to identify these breakdown products of DNA in the medium, from their characteristic ultraviolet spectrum.

We measured the electrophoretic mobility of the bacteria, in order to determine the magnitude of the charge per unit surface area [10]. The measurements were made immediately after irradiation, and 24 hours later, with exclusion of light (Fig. 1).

Such measurements were made in 1931 by Lisse and Tittsler [11], who reported, without attempting to explain their findings, that the electrophoretic mobility of the bacteria rose immediately after ultraviolet irradiation, and then fell, preceding death of the organisms.

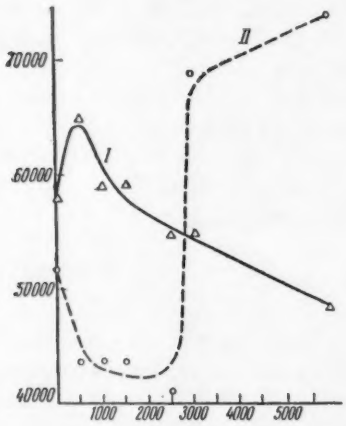

Fig. 1.

At the same time, the media in which the bacteria were suspended (after removal of the bacteria by centrifugation and filtration through asbestos)

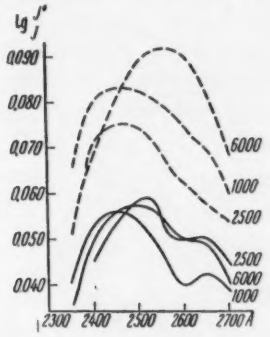

Fig. 2.

Fig. 1. Electrophoretic changes shown by Escherichia coli after exposure to various doses of ultraviolet light. Abscissae: intensity of irradiation, in ergs/mm^2, with ultraviolet light of wave length 2537 A. Ordinates: σ – density of charge per unit of surface area. I) Bacteria examined immediately after irradiation, II) bacteria kept in the dark for 24 hours after irradiation.

Fig. 2. Spectrophotometric examination of the medium of Escherichia coli suspensions, after irradiation with different doses (1000, 2500, and 6000 ergs/mm^2) of ultraviolet light of wave length 2637 A.(——) Medium examined immediately after irradiation, (------)medium from suspensions kept in the dark for 24 hours afer irradiation.

were examined spectrophotometrically, for the detection of desoxyribonucleic acid fragments, both immediately after irradiation and after keeping the irradiated suspensions in the dark for 24 hours (Fig. 2).

Our experiments were made on Escherichia coli, cultured on agar, and then in 1% peptone medium, for 24 hours at 37°. The bacteria were harvested by centrifugation, and washed three times on the centrifuge with acetate buffer (μ = 0.01 ; pH 5.5). Portions of bacterial suspension (20 ml, containing 4×10^9 cells per ml) were exposed to various doses of ultraviolet irradiation, from a bactericidal G.E. lamp. The irradiated suspensions were divided into 2 10-ml portions, one of the which was examined immediately, and the other was left in the dark for 24 hours. They were then passed through a Seitz filter and the filtrates were examined spectrophotometrically at 2300-2700 A; The electrophoretic mobility of the bacteria was examined in acetate buffer (μ = 0.01; pH 5.5). The density of charge per unit of surface in this buffer solution, containing only univalent ions, was calculated from the following formula [10]:

$$\sigma = 2\sqrt{\frac{NDkT}{2000\pi}}\,\mu \sin h \frac{\varepsilon}{2kT}.$$

The curves relating density of charge per unit of surface area to dosage of ultraviolet light indicate the following: a) the density of charge per unit area of the cells rises immediately after irradiation of the bacteria with small doses of ultraviolet light; with large doses there is competition between the effect of irradiation leading to increase in density of the surface charge (cleavage of peptide links, etc.), and that causing liberation of functional groups, and hence lowering the unit density of the surface charge (deamination, decarboxylation, etc.); and b) a marked fall in unit density of the surface charge of the same irradiated population of bacteria after it had been kept in the same suspension for 24 hours with exclusion of light.

Spectrophotometric analysis of the acetate buffer solutions in which the bacteria were suspended when irradiated showed the following: a) a uniform level of absorption at frequencies corresponding with those characteristic of nucleic acids, in media separated immediately after irradiation, and b) increase in optical density at these characteristic frequency bands when the suspensions had been kept in the dark for 24 hours after irradiation.

The electrophoretic examination of the bacterial suspensions which had been kept in the dark for a certain time after irradiation with ultraviolet light thus indicates the loss from the cells of those substances which caused increase in unit density of charge immediately after irradiation. Spectrophotometric examination of the medium in which the bacteria had been suspended showed that these substances had passed from the cells into the aqueous solution. The photoreactivation of the irradiated bacteria, which had been achievable during the first few minutes after exposure to ultraviolet light, because of the presence within the cells of the nucleic acid fragments, now became impossible, owing to passage of these depolymerization products into the aqueous medium. These observations provide evidence that the phenomenon of photoreactivation is due to the recombination of the fragments, to reconstitute desoxyribonucleic acid macromolecules.

SUMMARY

The density of charge per unit area of surface of Escherichia coli cells rises immediately after ultraviolet irradiation, as shown by measurements of electrophoretic mobility. It then falls, if the suspensions are kept in the dark for some hours. Spectrophotometric examination of the suspension media shows that they contain substances absorbing in the ultraviolet, at frequency bands characteristic of nucleic acids, and that the content of such products rises when the cells are kept in the dark. It is concluded that the immediate effect of ultraviolet irradiation is to cause depolymerization of desoxyribonucleic acids, the fragments of which are initially attached to the cells; photoreactivation of the cells is possible in this phase, owing to reconstitution of the macromolecules from the fragments. When the irradiated cells are stored in the dark, the depolymerization products pass out of the cells into the medium, and photoreactivation is then no longer possible.

Received July 18, 1957

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NATURE OF THE N-TERMINAL AMINO ACID RESIDUES OF WHEAT GLIADIN

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The identification of the terminal amino acid residues of proteins is one of the more important tasks of protein chemistry, at its present stage of development.

The determination of the molar proportions of amino and carboxyl groups at the ends of the peptide chains and as side groups of proteins permits, on the one hand, the drawing of conclusions relating to the number of polypeptide chains entering into the structure of a protein molecule, and provides evidence as to the presence or absence of branching of the chain. On the other hand, the elucidation of the nature of the terminal groups is the first step toward determining the sequence of the amino acid residues making up the peptide chains of which the protein macromolecule is constituted.

In continuation of our researches on the structure of proteins [1], we determined the amino acid composition of wheat and rye gliadins in 1955 [2], and established the presence in these prolamines of such amino acids as lysine, threonine, serine, and phenylalanine, about which there had previously been some doubt.

In view of the conflicting nature of the evidence published in the literature regarding the structure of the prolamines, we devoted the present research to the elucidation of the nature of the N-terminal groups of wheat gliadin.

EXPERIMENTAL

Gliadin was prepared from "Ukrainka" wheat; its amino acid composition had been determined by us in a previous paper [2]. We used Osborne's method, in Kiesel's modification, as described by us in this paper [2].

The N-terminal groups were identified by Sanger's method [3], by reacting α-amino groups with 1-fluoro-2,4-dinitrobenzene. The fluorodinitrobenzene was synthesized by the method of Zahn and Wurz [4].

About 300 mg of gliadin was dissolved in 70% ethanol, and 15 ml of 5% fluorodinitrobenzene in ethanol was added. The mixture was then shaken for 2 hours at room temperature, after adding 0.3 g of sodium bicarbonate. The precipitate of dinitrophenyl derivative of the protein (DNP-gliadin) was washed with ethanol, to remove unreacted fluorodinitrobenzene, and then with water, to remove sodium bicarbonate; the product was then treated with ether and dried. About 200 mg of DNP-gliadin was hydrolyzed with 6 ml of 6 N HCl in a sealed tube immersed in a boiling water bath for 6 hours. The hydrolyzate was separated from humins by filtration, the filtrate was freed of HCl by evaporation to dryness, and the residue was dissolved in 5 ml of water, and extracted 5 times with ether. The combined ether extracts were evaporated to dryness at room temperature. The residue, which contained the DNP-derivatives of the N-terminal amino acids, was dissolved in 1 ml of 2% aqueous sodium bicarbonate, and the solution was chromatographed on Volodarskii Leningrad Factory paper No. 1. The acid solution containing ether-insoluble DNP-derivatives was evaporated to dryness on a water bath, and the residue was dissolved in 2% bicarbonate. The solvent systems used for chromatography were: 1) citrate buffer (pH 6.2); 2) phenol – cresol (7:3), water-saturated; 3) toluene – pyridine – ethylene-

chlorohydrin – 0.8 N ammonia (10 : 10: 1: 1) ("toluene solvent").

According to Sanger [3], the ether extract of the acid hydrolyzate contains the DNP-derivatives of all the N-terminal amino acids except bis-DNP-histidine, DNP-arginine, and mono-ε-DNP-lysine (being in a side-group), which remain in the acid aqueous solution.

Both the ether-soluble and the ether-insoluble fractions were chromatographed. The chromatograms showed a row of yellow spots when we applied a drop (0.01 ml) of bicarbonate solution of the ether-soluble fraction of the hydrolyzate, and developed with the above solvents. With citrate buffer we obtained four spots, R_f 0.56, 0.68, 0.75, and 0.82 (means of ten determinations). Phenol – cresol solvent also gave 4 spots, while "toluene solvent" gave five. For the identification of the spots we determined the R_f values of some synthetic DNP-derivatives of known amino acids, with different solvent systems, at 18° (Table 1).

TABLE 1

R_f Values Given by DNP-Derivatives of Synthetic Amino Acids with Different Solvent Systems (means of 10 determinations)

DNP-derivative	"Toluene solvent"	Phenol – cresol	Citrate, pH 6.2	DNP-derivative	"Toluene solvent"	Phenol – cresol	Citrate, pH 6.2
DNP-glutamic acid	0.05	0.27	0.80	DNP-alanine	0.60	0.60	0.69
DNP-aspartic acid	0.10	0.32	0.82	DNP-proline	0.64	0.73	0.56
Mono-DNP-lysine	0.11	0.87	0.60	DNP-phenylalanine	0.68	0.90	0.66
DNP-arginine	0.12	0.94	0.64	DNP-leucine	0.75	0.89	0.74
bis-DNP-lysine	0.28	0.60	0.46	DNP-valine	0.77	0.79	0.70
DNP-cystine	0.30	0.40	0.52	bis-DNP-tyrosine	0.81	0.80	0.23
DNP-glycine	0.43	0.56	0.53	bis-DNP-histidine	0.84	0.87	0.15
DNP-serine	0.44	0.42	0.67	Dinitrophenol	0.66	0.41	0.56
DNP-threonine	0.48	0.64	0.76	Nitroaniline	0.98	0.96	—
DNP-methionine	—	0.55	0.70				

Our examination of the rate of movement of DNP-derivatives of synthetic amino acids in three different solvent systems showed that the R_f values varied very widely at different temperatures, and also depended on a number of other factors, such as time of run, distance from the starting point to where the paper dipped into the solvent, the distance between the starting point and the solvent front, and others; the magnitude of the R_f values cannot therefore be regarded as being altogether constant.

It is, however, possible to place the DNP-amino acids into slow- and fast-running groups, or to group the DNP-derivatives in the order of the approximate increase of R_f in a given solvent system.

Thus, in the chromatographic separation of 20 DNP-amino acids in citrate buffer we found that bis-DNP-histidine and bis-DNP-tyrosine were the slowest-moving. A large group of DNP-amino acids ran much faster: cystine, serine, glycine, phenylalanine, alanine, valine, proline, and methionine. The dinitrophenol spot should be added to this group. Fast-running spots in citrate solvent were: DNP-threonine, DNP-leucine, DNP-aspartic acid, and DNP-glutamic acid. One could thus readily distinguish the DNP-derivatives of tyrosine and histidine (in the citrate solvent system) from those of leucine, aspartic acid, and glutamic acid.

The opposite picture was seen with phenol – cresol solvent (Table 1). Here the slowest-running spots were those due to DNP-glutamic acid and DNP-aspartic acid, whereas DNP-tyrosine and bis-DNP-histidine moved fairly fast; this allowed the ready identification of these DNP-amino acids, using these two solvent systems.

However, in order to identify the DNP-derivatives running at intermediate speeds it was necessary to find their R_f values in a third solvent. It appeared from our experiments that the rate of movement of many of the DNP-derivatives varied considerably, in the same solvent system, often for no apparent reason. The

greatest variability in this respect was found for the DNP-derivatives of the intermediate group, viz., serine, cystine, methionine, glycine, threonine, alanine, and lysine.

These observations are evidence of the unreliability of basing identification of DNP-amino acids on comparisons of the R_f values with those of reference spots. The only trustworthy means of identification of DNP-derivatives of N-terminal amino acids is to compare the rates of movement of a given spot with that of reference spots developed in three or more different solvent systems. Reference spots which move at a different rate from the unknown should be excluded, and only those which move at the same rate in at least three different solvent systems should be taken into consideration.

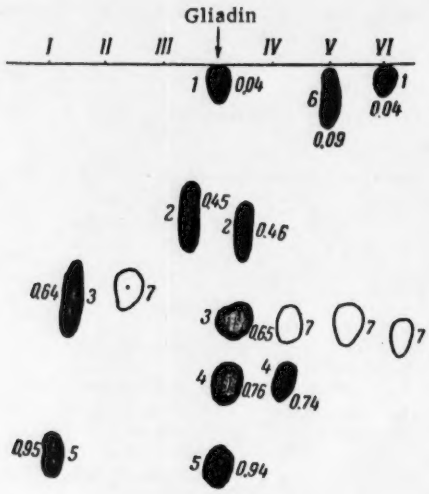

Fig. 1. Chromatogram of a hydrolyzate of a DNP-derivative of gliadin from "Ukrainka" wheat, after treatment with gaseous HCl ("toluene solvent"). Reference spots – DNP-derivatives of the amino acids; I) phenylalanine, II) dinitrophenol, III) threonine, IV) leucine, V) aspartic acid, VI) glutamic acid; 1) glutamic acid, 2) threonine, 3) phenylalanine, 4) leucine, 5) dinitroaniline, 6) aspartic acid, 7) dinitrophenol. In this and subsequent chromatograms the corresponding R_f values are indicated beside the given spot.

In accordance with the above, and by exclusion of reference spots which ran at different rates from the unknowns, we found that, using citrate buffer, spot 1 (R_f 0.56) of the chromatograms of the ether-soluble fraction of DNP-gliadin hydrolyzate corresponded with dinitrophenol (R_f 0.55), spot 2 (R_f 0.68) with DNP-phenylalanine (R_f 0.67), spot 3 (R_f 0.75) with DNP-threonine (R_f 0.76) or with DNP-leucine (R_f 0.74), and spot 4 (R_f 0.82) with DNP-glutamic acid (R_f 0.82) (Fig. 1). When phenol – cresol was used for development (Table 2) we found that the N-terminal amino acids of gliadin might be; 1) DNP-glutamic acid (R_f 0.29), corresponding with spot 1 (R_f 0.26), 2) DNP-threonine (R_f 0.66), corresponding with spot 3 (0.65), and 3) DNP-phenylalanine (R_f 0.92) and DNP-leucine (R_f 0.89), both corresponding with spot 4 (R_f 0.89). Spot 2 on the phenol – cresol chromatogram is due to dinitrophenol, which is a decomposition product of most of the DNP-amino acids.

The chromatograms of the ether-soluble fraction of DNP-gliadin hydrolyzate, developed in "toluene solvent", confirmed (see Table 2) that the N-terminal groups of DNP-gliadin are: 1) DNP-glutamic acid (R_f 0.05), corresponding with spot 1 (R_f 0.06), 2) DNP-threonine (R_f 0.50), corresponding with spot 2 (R_f 0.48), 3) DNP-phenylalanine (R_f 0.67), corresponding with spot 3 (R_f 0.68), and 4) DNP-leucine (R_f 0.75), corresponding with spot 4 (R_f 0.78). Spot 5 (R_f 0.96) corresponds with that due to dinitroaniline (Fig. 2). That spot 2 of the phenol – cresol chromatogram and spot 1 of the citrate chromatogram are due to artifacts is readily confirmed

by treating the chromatograms with gaseous HCl. According to Sanger [5], this treatment causes fading of the yellow dinitrophenol spot, while the DNP-amino acid spots assume a lemon-yellow shade.

Chromatograms of DNP-gliadin hydrolyzates after treatment with gaseous HCl, to remove spots due to dinitrophenol (and also dinitroaniline), are represented in Fig. 3.

As is evident from Fig. 3, three of the spots seen on the phenol – cresol and the citrate chromatograms correspond with four of the reference spots, DNP-leucine and DNP-threonine being at the same level as the middle spot of the citrate chromatogram, and DNP-leucine and DNP-phenylalanine at the same level as the lowest spot of the phenol – cresol chromatogram. The identification of those spots which correspond with two of the reference spots was achieved by "toluene solvent" chromatography, whereby each of the four spots corresponds with only one reference spot. The four DNP-amino acids shown in this chromatogram indicate that the N-terminal groups of gliadin are glutamic acid, threonine, phenylalanine, and leucine (Fig. 3). A visual appreciation of the intensity of staining of the four "toluene solvent" spots showed that it is about equal for the first three, but somewhat weaker for the DNP-leucine spots, indicating that the gliadin molecule contains fewer N-terminal leucines than N-terminal glutamic acid and

TABLE 2

R_f Values of the Ether-soluble Fraction of DNP-Gliadin Hydrolyzates and of the Corresponding Reference Spots, with Various Solvent Systems

Unknown spot		Reference spot	
No.	R_f	R_f	substance
		Citrate, pH 6.2	
1	0.56	0.55	Dinitrophenol
2	0.68	0.67	DNP-phenylalanine
3	0 75	0.74	DNP-leucine
		0.76	DNP-threonine
4	0.82	0.82	DNP-glutamic acid

Unknown spot		Reference spot	
No.	R_f	R_f	substance
		Phenol-cresol	
1	0.26	0.29	DNP-glutamic acid
2	0.54	0.53	Dinitrophenol
3	0.65	0.66	DNP-threonine
4	0.89	0.92	DNP-phenylalanine
		0.89	DNP-leucine

Unknown spot		Reference spot	
No.	R_f	R_f	substance
		"Toluene solvent"	
1	0.06	0.05	DNP-glutamic acid
2	0.48	0.50	DNP-threonine
3	0.68	0.67	DNP-phenylalanine
4	0.78	0 75	DNP-leucine
5	0.96	0.98	Nitroaniline

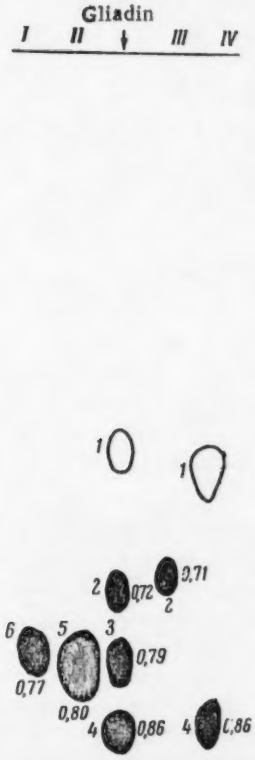

Fig. 2. Chromatogram of a hydrolyzate of DNP-derivative of "Ukrainka" wheat gliadin, after treatment with gaseous HCl (citrate buffer solvent, pH 6.2). Reference spots – DNP-derivatives of the amino acids: I) leucine, II) threonine, III) phenylalanine, IV) glutamic acid. 1) DNP, 2) phenylalanine, 3) threonine + leucine, 4) glutamic acid, 5) threonine, 6) leucine.

threonine residues. The yellow spot due to phenylalanine on the "toluene solvent" chromatogram became somewhat paler when treated with gaseous HCl, but it still retained a definite yellow color, suggesting that traces of dinitrophenol were present in this spot, as would be expected from the closeness of its R_f value (0.66). Chromatography of the ether-insoluble fraction of the hydrolyzate showed that it contained some ϵ -mono-DNP-lysine. The chromatograms, developed in phenol – cresol and citrate solvent systems, showed only a single yellow spot, turning rose-brown after spraying with ninhydrin. It follows that the fraction contained ϵ -DNP-derivative of lysine side-chains, and that N-terminal histidine and arginine were absent from it.

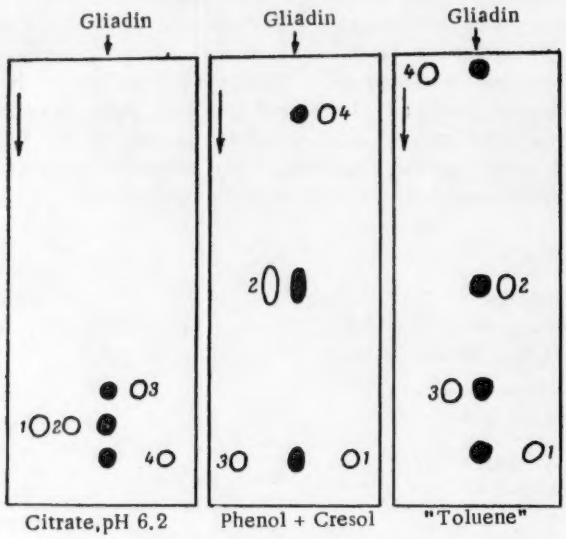

Fig. 3. Chromatograms of DNP-gliadin hydrolyzates, developed in three different solvent systems, and after treatment with gaseous HCl. Reference spots – DNP-derivatives of the amino acids: 1) leucine, 2) threonine, 3) phenylalanine, 4) glutamic acid.

DISCUSSION

The literature references to the N-terminal amino acids of wheat gliadin contain very conflicting data.

Thus according to Koros [6], the N-terminal amino acids of Hungarian wheat gliadin are histidine residues. According to Ramachandran and MacConnell [7] Canadian wheat gliadin N-terminal groups include histidine (3 molecules), with smaller amounts of aspartic and glutamic acids, alanine, valine, and serine. Deutsch and Sorenyi [8], using the fluorodinitrobenzene method, reported that the N-terminal residues of Hungarian wheat gliadins are phenylalanine groups. Devenyi and Sorenyi [9] report similar results for Hungarian wheats. Mills [10] fractionated DNP-derivatives of wheat gliadin in 1955, and isolated water-soluble fractions of DNP-gliadin; he was able to identify aspartic acid, serine, and valine in the complex mixture of 15 DNP-amino acids shown by chromatography.

The reason for the divergent results reported by these authors appears to lie in the experimental difficulties of the chromatographic analysis of DNP-amino acids. First, the DNP-amino acid spots are very frequently masked by those due to their breakdown products, dinitrophenol and dinitroaniline, which are very difficult to eliminate. Secondly, the rate of movement of the DNP-amino acids is affected by very many factors (not only temperature, but also by the sort of paper used, by the time elapsed since preparation of the solvent systems, by the degree of saturation with water vapor of the atmosphere within the chromatography tank, by the time taken for development of the chromatograms, by the distance from the starting point to the level of the solvent, by the distance from the starting point to the solvent front, by the amounts of DNP-amino acids taken for analysis, and by the presence of dinitrophenol and dinitroaniline), which affect the rate of movement of DNP-amino acids, as well as by a number of other factors, which cannot sometimes even be evaluated.

Biserte and Osteux [11] have reported some of the factors affecting the magnitude of the R_f values of DNP-amino acids. According to these authors, the R_f values may differ by 100%, according to the degree of saturation with water vapor of the atmosphere of the chromatography tank. The distance of the solvent front from the starting point, and the amounts of DNP-amino acids taken fro the spots also had considerable effects on the R_f values.

Our conclusions regarding the nature of the N-terminal amino acids of gliadin are based on the coincidence of the rates of movement of the reference and the unknown spots in three different solvent systems. Only in this way were we able to arrive at sufficiently trustworthy conclusions that the N-terminal amino acids of wheat gliadin are glutamic acid, threonine, phenylalanine, and leucine. The divergencies between our findings and those of Koros [6] and Ramachandran and MacConnell [7] may be ascribed to the faulty interpretation given by these authors to their chromatograms of the water-soluble fraction of DNP-gliadin hydrolyzates. In the given case, on the chromatograms of the ether-insoluble fraction (which could have contained terminal DNP-arginine, bis-DNP-histidine, and mono-DNP-lysine), developed in phenol – cresol solvent, only mono-DNP-lysine was actually present; its R_f coincides with that of bis-DNP-histidine (0.87 in both cases) (see Table 1). Under such circumstances, the yellow spot due to mono-DNP-lysine may easily be taken to be that due to bis-DNP-histidine, and this is probably what happened in the above-cited researches. However, the actual identity of the single spot found on the chromatograms of the ether-insoluble fraction could be readily established by spraying with ninhydrin, as was done by us in an earlier research [12]. This caused a change in the color of the spot, from yellow to rose-brown, showing that mono-DNP-lysine (but not DNP-histidine) was present, which because of to its free amino group gave the brownish-red color.

The above-cited authors [7] found that the ether-soluble fraction of the hydrolyzate contained DNP-glutamic acid, in small amount, and our work confirms these findings. In addition, these authors reported the presence of the DNP-derivatives of alanine, valine, and serine in this fraction. We would comment, in this connection, that the R_f values of these DNP-amino acids are fairly close to that of dinitrophenol, which may explain the erroneous identification of these spots made by the authors in question. Similar comments would also apply to the work of Mills [10]. The findings of Deutsch and Sorenyi [8] and of Devenyi and Sorenyi [9], that the N-terminal groups of wheat gliadin consist of phenylalanine only, are supplemented by our additional finding of other N-terminal amino acids.

We would, in conclusion, draw attention to the possibility of drawing erroneous conclusions as to the identity of the N-terminal amino acids of proteins, due to the uncertainty of the methods available, and to the presence of interfering nitro-derivatives; the present research was to a large extent devoted to the overcoming of these difficulties.

SUMMARY

Sanger's method was applied to the identification of the N-terminal amino acids of wheat gliadin, treated with fluorodinitrobenzene. The terminal DNP-amino acids were identified by means of paper partition chromatography, using three different solvent systems. These were: 1) citrate buffer solution, pH 6.2, 2) water-saturated phenol – cresol (7 : 3), and 3) toluene - pyridine – ethylene chlorohydrin– 0.8 N ammonia (10: 10: 1: 1). Twenty different DNP-amino acids were synthesized, for use as reference spots. The identity of unknown spots with reference spots was accepted if they moved at the same rates in all three solvent systems. The ether-soluble fraction of the DNP-gliadin hydrolyzate was thus shown to contain glutamic acid, threonine, phenylalanine, and leucine.

The chromatogram of the ether-insoluble fraction of the hydrolyzate showed only one spot, due to mono-DNP-lysine. This could be distinguished from any other product by the rose-brown coloration given with ninhydrin, unlike any other DNP-amino acid of similar R_f value.

The N-terminal amino acids of wheat gliadin are therefore glutamic acid, threonine, phenylalanine, and leucine. Lysine is present in the peptide chain.

Received July 23, 1957

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SPECTROPHOTOMETRIC DETERMINATION OF TOTAL NUCLEIC ACID CONTENT

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The most useful method of determination of nucleic acids is that based on ultraviolet spectrophotometry. This method is distinguished by its exceptional simplicity, rapidity, and accuracy. It is, at the same time, more sensitive than are other methods.

An essential stage of all the ordinarily applied spectrophotometric methods for the determination of nucleic acids is their extraction from the material. This extraction involves some form of standard hydrolysis of the nucleic acids, such as alkaline hydrolysis according to Schmidt-Thannhauser [1], or acid hydrolysis according to Ogur and Rosen [2], or to Schneider [3]. The necessity of introducing the standard hydrolysis step is due to the circumstance that absorption in the ultraviolet varies for unhydrolyzed nucleic acids, depending on their structure, and on the pH and ionic strength of the medium, etc.

Differential extraction is nearly always practiced for the ultraviolet spectrophotometry of nucleic acids, giving the RNA and DNA fractions separately [2, 4, 5]. The optical density of each fraction is measured at the absorption maximum of the corresponding nucleic acid, being 260 mμ for RNA hydrolyzates, and 268 mμ for DNA hydrolyzates [2, 4]. The amount of the given nucleic acid present in the extracts is calculated from the optical density found at these frequencies.

It is, however, often necessary, in working with nucleic acids, to know the total nucleic acid content of a tissue, i.e., to determine its DNA + RNA content, and for this a rapid and accurate micromethod is needed. Methods depending on determination of purine nitrogen are laborious and inaccurate, while those based on phosphorus determination may give misleading results, owing to the presence of other phosphorus-containing substances. Summation of the results found separately, by a spectrophotometric method, for the DNA and RNA contents gives the most accurate results, but the preliminary separation procedure is somewhat time-consuming We have for these reasons attempted to find conditions that would permit the determination of the total nucleic acid content of a sample, by direct spectrophotometry of the appropriate extract.

Of the methods used for extraction of nucleic acids, with their simultaneous standard hydrolysis, that depending on hot acid treatment of the material is the most widely applicable. Schneider's method [3], involving the treatment of the material with trichloroacetic acid (TCA) at 90°, is one of the best and most widely tested methods, assuring the rapid and quantitative extraction of nucleic acids from any material. It is not, however, applicable to direct spectrophotometry, because of the strong absorption of TCA itself, in the 240-300 mμ region, although there are references in the literature to attempts at applying it to the determination of nucleic acids [6]. It was shown by Ogur and Rosen [2] that hot extraction with perchloric acid is as effective as extraction with TCA. Similarly to TCA, perchloric acid is a specific protein precipitant, so giving protein-free extracts. It has the advantage over TCA of not absorbing in the ultraviolet, at the absorption maxima of nucleic acid (240-300 mμ).

On this basis, we extracted total nucleic acids by heating the sample with 0.5 N perchloric acid for 20 min in a boiling water bath. This procedure ensured the standard acid hydrolysis of nucleic acids to soluble products. It was shown by special tests that this procedure always gives quantitative extraction of nucleic acids from tissues.

In order to find the relation between the optical density of such extracts in the ultraviolet and their nucleic acid content, we subjected accurately weighed samples of standard preparations of thymus DNA and yeast RNA to hydrolysis under the above-specified conditions. The absorption spectra of the resulting hydrolyzates were recorded in the region from 240 to 320 mμ intervals, in quartz cuvettes with a 1 cm light path in a SF-4 spectrophotometer with a hydrogen light source, reading against a 0.5 N $HClO_4$ blank. On the basis of the phosphorus content of the standard preparations of RNA and DNA taken for hydrolysis we converted all the optical densities found for each frequency to the same standard concentration of nucleic acid phosphorus in the solution, viz., 1 μg of phosphorus per ml. The optical densities thus derived for 1 cm layers of solution containing 1 μg of nucleic acid phosphorus per ml were designated as specific extinctions, $E^{\mu gP}$ (for RNA, $E^{\mu gP\text{-}RNA}$, and for DNA $E^{\mu gP\text{-}DNA}$). In the figure, the values found for specific extinction are plotted against the corresponding wave lengths, for yeast RNA and thymus DNA hydrolyzates. Insepection of curves 1 and 2 of the graph shows that specific extinctions of RNA hydrolyzates are greater than those of DNA hydrolyzates, over the whole range of frequencies. Thus the curves do not intersect at any point, showing that there is no extinction coefficient common to both RNA and DNA, and that it is not possible from the specific extinctions to arrive at a trustworthy value for the sum of the DNA and RNA contents of a mixture. The total optical density of a mixed hydrolyzate would depend not only on the concentrations of the separate components, but also on their relative proportions in the mixture.

Thus at a given wave length λ the total optical density D_λ of the mixed extract (RNA + DNA) will be the sum of the optical densities of each component taken separately:

$$D_\lambda = D_\lambda^{RNA} + D_\lambda^{DNA} \qquad (I)$$

In turn, the optical density of each component is directly proportional to its concentration and to its specific extinction (Beer's law):

$$D_\lambda^{RNA} = E_\lambda^{RNA} \cdot C^{RNA} \; ; \; D_\lambda^{DNA} = E_\lambda^{DNA} \cdot C^{DNA}$$

Combining the two, we may write:

$$D_\lambda = E_\lambda^{RNA} \cdot C^{RNA} + E_\lambda^{DNA} \cdot C^{DNA} \qquad (II)$$

In our case, RNA and DNA concentrations are expressed as micrograms of phosphorus per ml, and equation II takes the form:

$$D_\lambda = E_\lambda^{\mu gP\text{-}RNA} \cdot C^{\mu gP\text{-}RNA} + E_\lambda^{\mu gP\text{-}DNA} \cdot C^{\mu gP\text{-}DNA} \qquad (IIa)$$

where D_λ is determined directly be spectrophotometry at wave length λ, $E_\lambda^{\mu gP\text{-}RNA}$ and $E^{\mu gP\text{-}DNA}$ are read from the curves for standard preparations (Graph, curves 1 and 2), and $C^{\mu gP\text{-}RNA}$ and $C^{\mu gP\text{-}DNA}$ are unknowns. Only in the case

$$E_\lambda^{\mu gP\text{-}RNA} = E_\lambda^{\mu gP\text{-}DNA}$$

i.e., in the case of the intersection of the specific extinction curves of RNA and DNA at any wave length λ, can equation IIa by solved, taking $C^{\mu gP\text{-}RNA} + C^{\mu gP\text{-}DNA}$ to be the required total nucleic acid content, in an equation with one unknown, $C^{\mu gP\text{-}sum}$:

$$D_\lambda = E_\lambda^{\mu gP} \cdot C^{\mu gP\text{-}sum}$$

However, over the whole length of the characteristic nucleic acid spectrum (240-300 mμ)

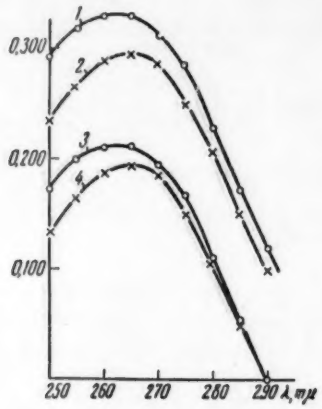
$$E_\lambda^{\mu gP\text{-}RNA} > E_\lambda^{\mu gP\text{-}DNA}$$

We overcame this difficulty in solving the problem of determining the total nucleic acid content of a mixture by applying the method of extinction differences ("Δ method"), widely applied in spectrophotometry

for minimizing the effects of unspecifically absorbing contaminants [7]. By this procedure, the calculations are based not on the absolute values for optical density and specific extinction, but on the difference between optical densities (and, correspondingly, specific extinctions) at two wave lengths, λ_1 and λ_2. Thus, if on the same graph (see figure) we plot against wave lengths the differences between the specific extinctions at that wave length and that at 290 mμ, i.e., the values

$$\Delta E_\lambda = E_\lambda - E_{290},$$

we obtain the ΔE curves 3 and 4. Inspection of these curves shows that the values of ΔE nearly coincide for RNA and DNA over the range 290 – 270 mμ.



Absorption spectra of standard preparations of nucleic acids.

1) $E^{\mu gP\text{-}RNA}_{\lambda}$; 2) $E^{\mu gP\text{-}DNA}_{\lambda}$;

3) $E^{\mu gP\text{-}RNA}_{\lambda - 290}$; 4) $E^{\mu gP\text{-}DNA}_{\lambda - 290}$.

——·——· yeast RNA

——x——x calf thymus DNA

If, in accordance with this procedure, we apply the general equations II or IIa, we obtain the following expressions:

$$D_\lambda = E^{\mu gP\text{-}RNA}_{\lambda} \cdot C^{\mu gP\text{-}RNA} + E^{\mu gP\text{-}DNA}_{\lambda} \cdot C^{\mu gP\text{-}DNA}$$

$$D_{290} = E^{\mu gP\text{-}RNA}_{290} \cdot C^{\mu gP\text{-}RNA} + E^{\mu gP\text{-}DNA}_{290} \cdot C^{\mu gP\text{-}DNA}$$

$$D_\lambda - D_{290} = (E^{\mu gP\text{-}RNA}_{\lambda} - E^{\mu gP\text{-}RNA}_{290}) \cdot C^{\mu gP\text{-}RNA} + (E^{\mu gP\text{-}DNA}_{\lambda} - E^{\mu gP\text{-}DNA}_{290}) \cdot C^{\mu gP\text{-}DNA}.$$

Writing ΔD_λ for $D_\lambda - D_{290}$, and ΔE_λ for $E_\lambda - E_{290}$, this equation takes the form:

$$\Delta D_\lambda = \Delta E^{\mu gP\text{-}RNA}_{\lambda} \cdot C^{\mu gP\text{-}RNA} + \Delta E^{\mu gP\text{-}DNA}_{\lambda} \cdot C^{\mu gP\text{-}DNA} \qquad \text{(III)}$$

It is, however, evident from curves 3 and 4 of the graph that at wave lengths of 270 mμ up, $\Delta E^{\mu gP\text{-}RNA}_{\lambda} \simeq E^{\mu gP\text{-}DNA}_{\lambda}$. Then, taking the highest specific extinction at which this equation still holds, viz., specific extinction at λ = 270 mμ, we obtain the equation:

$$\Delta D_{270} = \Delta E^{\mu gP}_{270} \cdot (C^{\mu gP\text{-}RNA} + C^{\mu gP\text{-}DNA}), \text{ i.e., } C^{\mu gP\text{-}sum} = \frac{\Delta D_{270}}{\Delta E^{\mu gP}_{270}} \qquad \text{(IV)}$$

In this way, by calculating the difference between the optical densities at 270 mμ and at 290 mμ ($D_{270} - D_{290} = \Delta D_{270}$) of the solution under examination, and taking for the calculation the corresponding difference between the specific extinctions, it is possible to derive the total nucleic acid concentration of the extract, with a fairly high accuracy. We found that, for hydrolyzates of standard preparations of yeast RNA and thymus DNA, ΔE_{270} is approximately equal to 0.19.

$$(\Delta E_{270}^{\mu gP\text{-}RNA} = E_{270}^{\mu gP\text{-}RNA} - E_{290}^{\mu gP\text{-}RNA} = 0.314 - 0.121 = 0.193;$$

$$\Delta E_{270}^{\mu gP\text{-}DNA} = E_{270}^{\mu gP\text{-}DNA} - E_{290}^{\mu gP\text{-}DNA} = 0.287 - 0.101 = 0.186;$$

in round figures, $\Delta E_{270} = 0.19$).

We proposed the following very simple variant of the spectrophotometric method for the determination of total nucleic acid content of tissues, nucleoproteins, protein fractions, etc. A weighed sample of material (from 5 to 500 mg) is added to an exactly measured amount (5-10 ml) of 0.5 N $HClO_4$, and the mixture is heated on a boling water bath for 20 min. The solution is then cooled and centrifuged, and the supernate is taken for spectrophotometry, either without dilution, when the nucleic acid concentration is expected to be low, or after appropriate dilution with 0.5 N $HClO_4$. Absorption is measured at 270 and 290 mμ, taking 0.5 N $HClO_4$ as the blank, in 1 cm quartz cuvettes. The difference between the optical densities at 270 mμ and at 290 mμ is taken for the calculation. Dividing this difference by 0.19 gives the number of micrograms of nucleic acid phosphorus per ml. of solution.

$$C^{\mu gP\text{-}NA} = \frac{D_{270} - D_{290}}{0.19}$$

In order to convert the amount of nucleic acid phosphorus into the amount of nucleic acid itself, we use the usual average conversion factor of 10.3 [8].

$$C^{NA} = 10.3 \cdot C^{\mu gP\text{-}NA}.$$

The method also serves for the determination of the nucleic acid content of material containing only RNA or only DNA. However, in converting from nucleic acid phosphorus to nucleic acid it is preferable to use a conversion factor of 10.5 for RNA and of 10.1 for DNA (based on the theoretical phosphorus contents of 9.5% for RNA and 9.9% for DNA).

We recommend an additional, control measurement at 260 mμ. The optical density at 260 mμ should not differ greatly from that at 270 mμ (by not more than $\pm$ 15%). Divergencies exceeding $\pm$15% indicate the presence of considerable admixtures of non-nucleic acid contaminants. In such cases it sometimes helps to give the sample a preliminary wash with cold 0.2 N $HClO_4$. If this does not remove the impurities the temperature of extraction with 0.5 N $HClO_4$ may be reduced to 70°, and the time of extraction to 10 min, but the completeness of extraction should in that case be checked.

The method gives a fairly high degree of accuracy, and allows the very rapid serial determination of nucleic acid content of a wide range of materials. It differs from other spectrophotometric methods first in that it makes use of differences between extinctions at two different wave lengths, instead of direct use of extinction coefficients, and secondly, in that the readings are made not at the absorption maximum, but at 270 mμ. The use of the difference $E_{270} - E_{290}$ has the advantage that this value is very similar for both RNA and DNA; this permits of their joint determination, irrespective of their relative proportions in the mixture. Apart from this, the use of this value greatly reduces the effect of protein admixtures, since these have approximately the same absorption at 270 and 290 mμ, and it is thus cancelled out. Moreover, the differential extinction method largely eliminates the effects of unspecific absorption by the extract, of differences in absorption and cleanness of the cuvettes, of slight opalescence of the solutions, and of irregularities in the zero point of the spectrophotometer, etc.

A comparison of the results obtained using our method with those given by other methods is given in the table. Phosphorus was determined gravimetrically by Embden's method, purine nitrogen by the method of Graf and MacColl, pentoses by Youngberg's method, in Belozerskii's or Mejbaum's modification, and DNA by Dische's diphenylamine reaction [8].

We would add that the method gives quite good results for the determination of nucleic acids directly in biological material, such as animal and plant tissues, bacterial cells, etc. In such cases, however, more accurate results are obtained when the material is first washed free of acid-soluble constituents with cold 0.2 N $HClO_4$.

The most important limitation of the application of our method is that it gives considerable errors with nucleic acids of unusual nucleotide composition (when using the factor $\Delta E_{270} = 0.19$). The reservation applies chiefly to desoxyribonucleic acid preparations from certain microorganisms, with pronounced guanine – cytosine

or adenine – thymine types of DNA [9, 10] and to viruses, which are known to have a very specific nucleotide composition [11]. Our experiments showd that, taking $\Delta E_{270} = 0.19$, considerably lower results were obtained for DNA content of preparations from actinomycetes and from some bacteria (For example, Pseudomonas aeruginosa, Alkaligenes fecalis). The error amounts to 35% in the case of actinomycetes DNA. It may be concluded from published data on the composition of the DNA of a number of forms that the method of calculation based on the value of 0.19 for the factor ΔD_{270} would give low results for the DNA of actinomycetes [10, 12], of bacteria belonging to the genera Sarcina, Mycobacterium, Pseudomonas, and Alkaligenes [9, 10], and of molluscs and echinoderms [13], i.e., of organisms having a high guanine – cytosine type of DNA. Conversely, high results would be expected for organisms with a high adenine – thymine type of DNA, such as Clostridium, Staphylococcus, Streptococcus, Pasteurella, Micrococcus, and Bacillus [9,10]. The reason for these effects is that the extinction

TABLE

Percentage Content of Nucleic Acids, as Determined by Different Methods

Material analyzed	From P content	From carbohydrate part			From purine nitrogen content	Spectrophotometrically
		DNA	RNA	total nucleic acid		
Artificial nucleoproteins						
RNA-protein (from edestin + yeast RNA)	12.49	—	13 73	13.73	—	13.38
DNA-protein (guanidated edestin + bull DNA)	51.45	50.75	—	50.75	—	52.46
Ribonucleoproteins extracted from yeast						
Precipitated at pH 1	79.41	—	81.63	81.63	—	76.00
" at pH 4	—	—	4.28	4.28	—	4 84
" by alcohol	—	—	26.44	26.44	—	26.16
Bacterial desoxyribonucleoproteins						
Escherichia coli	—	35.50	—	35.50	—	32.81
Morgan's bacillus	—	8.01	—	8.01	—	8.50
Proteus vulgaris	—	9.60	—	9.60	—	10.47
B. fecalis alkaligenes	—	20.82	—	20.82	—	18.72
Mixed bacterial nucleoprotein fractions						
0.14 M NaCl extract of E. coli	34.39	4.44	26 19	30.63	39,02	36.50
M NaCl extract of E. coli	62.50	13.10	43.00	56.10	60,40	61,59
Whole bacterial cells						
Morgan's bacillus	—	5.06	5.60	10.66	—	10.78
Proteus vulgaris	—	5.34	4.48	9.82	—	10.03
Nucleoprotein from cedar nuts	26.17	21.57	3.76	25.33	—	25 91

at 290 mμ is due basically to the guanine and cytosine components of nucleic acids, and is therefore much higher for nucleic acids with a high content, and lower for those with a low content, of these bases. It follows that the actual value of $\Delta E_{270} = E_{270} - E_{290}$ would be less than 0.19 for desoxyribonucleic acids of the high guanine–cytosine type, and more than 0.19 for those of the high adenine – thymine type.

In view of the very close similarity in nucleotide composition of the ribonucleic acids of all cellular organisms (i.e., excluding viruses) [10], it may be considered, with a high degree of probability, that calculations based on a value of 0.19 for ΔE_{270} should give acceptable results in all (or nearly all) cases. The same appears to apply to the desoxyribonucleic acids of the higher plants and of most animals (including all classes of vertebrates), since these all have similar DNA compositions [14].

Thus our method, using the value of 0.19 for the factor ΔE_{270} for the calculation of the total nucleic acid content of the tissues of most animals and higher plants, as well as of a number of bacteria, is undoubtedly

applicable to such material. It is also applicable to a wider range of bacteria, and even of actinomycetes [15], when preparations containing mostly RNA are taken.

SUMMARY

A simple spectrophotometric method for the determination of the sum of the ribo- and desoxyribonucleic acid contents of biological material is described. It depends on the extraction of the material with hot 0.5 N perchloric acid, centrifuging, and determining the absorption of the supernatant solution at 270 mμ and at 290 mμ. The total nucleic acid content, in micrograms of nucleic acid phosphorus per ml of solution, is calculated by dividing the difference between the optical densities at 270 and 290 mμ by the factor 0.19 ($C^{\mu gP\text{-}NA} = \frac{D_{270} - D_{290}}{0.19}$).

The factor 0.19 is applicable only to nucleic acids of the usual nucleotide composition; reliable results are not obtained for nucleic acids of viral origin, or for those of a number of classes of microorganisms with very high or very low guanine–cytosine or adenine–thymine types of nucleotide composition.

The author wishes to thank N. A. Kokurina and L. P. Gavrilova for their assistance in working out the method, and also S. O. Uryson, B. F. Vaniushin, and N. V. Shugaeva for providing some of the analytical data.

Received September 27, 1957

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THE PROBLEM OF THE METHOD OF SEPARATION OF NUCLEIC ACIDS COMBINED IN VARIOUS FORMS WITH THE PROTEINS OF ANIMAL TISSUES

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At the present time it has been conclusively shown that the nucleic acids (RNA and DNA) have a high degree of specificity [1-4].

Since the nucleic acids (NA) can give complex compounds with proteins, it is of great interest to seek for a method of showing the particular specificity not only of the free nucleic acids but also of the nucleoproteins (NP). The difficulty of such a study is that until now there has been no method of separating the nucleoproteins in animal organs and tissues.

There has so far been little work in this direction. Some years ago [5] we showed that in the tissue of tumors there is a specific nucleoprotein in which the nucleic acid (DNA) compound with protein is rich in the amino acid tryptophan, covalently bound.

The work of Belozerskii [6, 7] showed experimentally that nucleic acids could give labile and stable complex compounds with proteins.

When we studied the physicochemical properties of some nucleoproteins isolated from tumors, we expressed the idea of the possible existence in these molecules of covalent "stable" bonds between the amino groups of adenine and the carboxyl groups of the aromatic amino acids [8, 9].

Further, using the Belozerskii method of fractionating nucleoproteins, we showed that nucleic acids combined by labile and covalent "stable" bonds with protein exchange their phosphorus residues at different rates in liver tissue [10].

When we studied this question in other animal tissues we were struck with the various difficulties of method.

As is known, the separation of "stable" and labile compounds of NA with proteins by the Belozerskii method is based on the extraction of NA from the "labile" NP by picric acid; the NA stably bound to proteins then remains in the NP. We should say that many valuable results have been obtained with this method. However, for various animal proteins it cannot be fully used, chiefly because of the loss of part of the nucleic acids during fractionation.

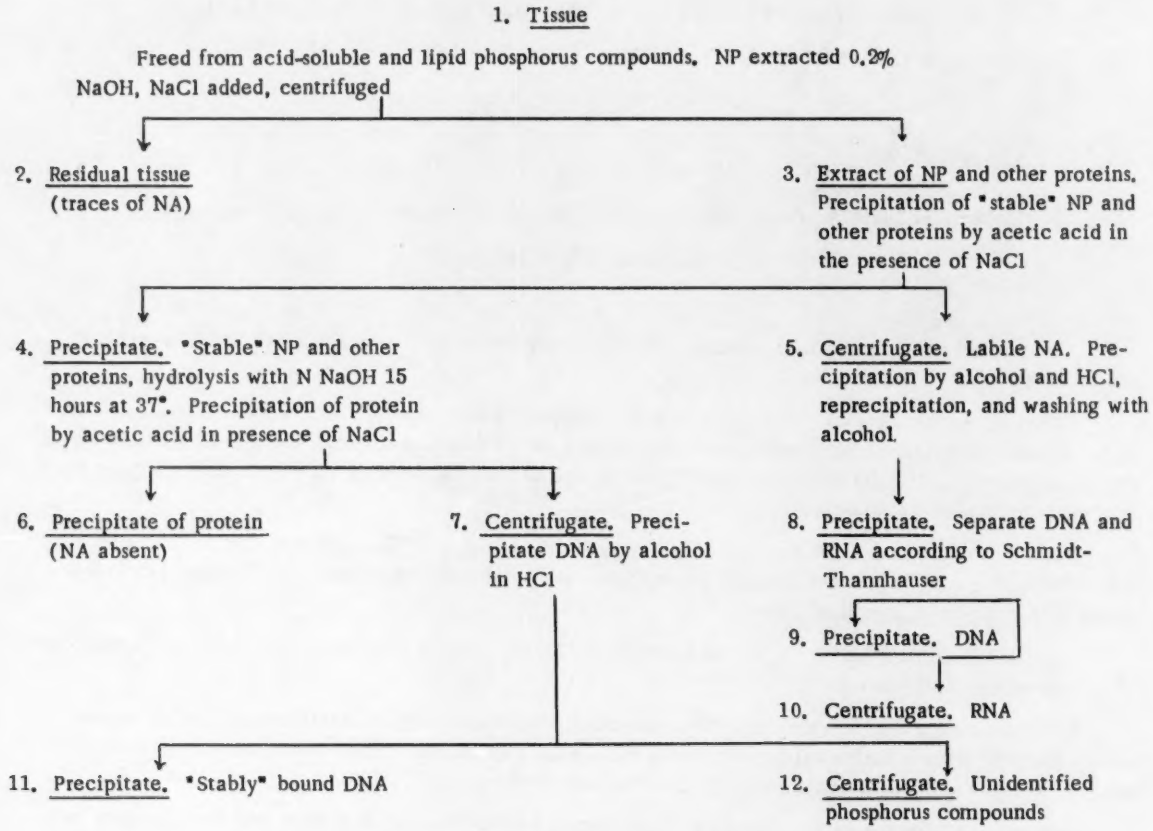
We therefore undertook a study to find other methods of separating NA.

EXPERIMENTAL PART

As a result of our attempts we found that part of the NA is precipitated with the protein from an alkaline extract of the tissue in the presence of NaCl by acetic acid while another part of the NA remains in solution. We assumed that the part of the NA which precipitates is the "stable" compound with the protein in the NP molecule, and the NA which remains in solution comes from the "labile" NP which is split under these conditions.

We tested our idea by experiments with repeated fractionations. We showed that after repeated fractionations 81-87% of the "stable" NA remained bound to protein. A supplementary yield of free NA composed 2-5% of the total amount of tissue NA, while after one fractionation the yield of "labile" NA was 74-87%.

On the basis of this principle we worked out a method for separating the nucleic acids bound to proteins in a "stable" or labile manner.


1. Tissue
Freed from acid-soluble and lipid phosphorus compounds. NP extracted 0.2% NaOH, NaCl added, centrifuged
2. Residual tissue (traces of NA)
3. Extract of NP and other proteins. Precipitation of "stable" NP and other proteins by acetic acid in the presence of NaCl
4. Precipitate. "Stable" NP and other proteins, hydrolysis with N NaOH 15 hours at 37°. Precipitation of protein by acetic acid in presence of NaCl
5. Centrifugate. Labile NA. Precipitation by alcohol and HCl, reprecipitation, and washing with alcohol
6. Precipitate of protein (NA absent)
7. Centrifugate. Precipitate DNA by alcohol in HCl
8. Precipitate. Separate DNA and RNA according to Schmidt-Thannhauser
9. Precipitate. DNA
10. Centrifugate. RNA
11. Precipitate. "Stably" bound DNA
12. Centrifugate. Unidentified phosphorus compounds

Scheme for Separation of Stable and Labile Compounds of Nucleic Acids with Proteins in Sodium Chloride Solutions.

METHODS

The tissues, frozen by liquid air, were ground in a mortar with 5% trichloroacetic acid, the suspension was transferred quantitatively to a centrifuge tube and left in the cold (snow, ice) for 20 min to extract the acid-soluble phosphorus. The samples were centrifuged and the centrifugate poured off. The extraction of the acid-soluble phosphorus compounds was repeated three times. Lipide compounds of phosphorus were removed by successive washings: three times with 96% alcohol, twice with ether, twice with alcohol. The residual tissue was mixed with water and the suspension was treated with a 2% solution of sodium hydroxide to a final concentration of 0.2%. Extraction of NP lasted for 20-30 min.

The extract was treated with 0.6 volume of saturated sodium chloride solution, the sample was carefully mixed and centrifuged. In the precipitate (2) was the insoluble tissue residue which contained traces of NA. In the centrifugate (3) were nucleoproteins and other proteins. The centrifugate was treated with 0.2 volume (of the starting extract) of 4 N acetic acid, whereupon "stable" NP and other proteins precipitated (4), and the "labile" nucleic acid remained in solution (5). The sample was centrifuged, the centrifugate was

poured into a tube, and the labile NA was precipitated by adding equal volumes of alcohol and hydrochloric acid to an acid reaction to Congo in 1-2 hours. The precipitate was centrifuged and the NA was purified from remaining admixtures by again dissolving in weak sodium hydroxide and precipitating with alcohol and hydrochloric acid. The "labile" was twice washed with alcohol.

Fraction (4) contained, besides Na "stably" bound to protein, considerable amounts of other substances which contained phosphorus.

To extract the NA in pure form, fraction (4) was once washed with water and subjected to hydrolysis with N NaOH in a thermostat at 37° for 15 hours. The sample was removed from the thermostat, cooled, and partly neutralized with 4 N hydrochloric acid (1 ml of hydrochloric acid to 6 ml of hydrolyzate), then the sample was treated with sodium chloride (800 mg NaCl per 6 ml of hydrolyzate), which was dissolved by stirring with a glass rod. The solution was treated with 4 N acetic acid (2 ml per 6 ml of hydrolyzate). Then a protein precipitated (6) which contained no NA; the nucleic acids which split from the protein during the hydrolysis remained in solution (7). The sample was centrifuged, the centrifugate was poured into a tube, and the NA which it contained was precipitated by adding an equal volume of alcohol, and hydrochloric acid to an acid reaction to Congo. After 1-2 hours the sample was centrifuged, centrifugate (12) which contained traces of RNA and other phosphorus compounds was discarded. Precipitate (11) was washed three times with 5% trichloroacetic acid to remove residual acid-soluble phosphorus compounds. The precipitate was DNA which was combined with protein through stable bonds.

This method produced separation of "stable" and labile compounds of NA with proteins from liver, kidney, spleen, and small intestine of the white rat.

The NA content in these preparations was determined by two different methods: by phosphorus (by the method of Fiske-Subbarow) and by reaction for the carbohydrate component (with diphenylamine for desoxyribose and with orcinol for ribose).

RESULTS

It was shown (Table 1) that liver contains on the average 97.5-102 mg of phosphorus of "stable" DNA per g of fresh tissue, 92-101 mg of phosphorus of "labile" DNA, and 574-588 mg of phosphorus of RNA; for kidney the corresponding figures are 106, 101-104, and 228-237; for spleen, 234-247, 401-422, and 325-338; for small intestine, 197-204, 251-267, and 436-442. Thus, the greater part of the nucleic acids of all the organs studied are united to protein through labile bonds, and in the composition of the labile NA both RNA and DNA are found; "stable" NA contains only desoxyribonucleic acid. Most of the "stable" DNA is found in the spleen and small intestine, and less in the liver and kidney.

Table 1 also shows that the differences between data obtained by reaction with diphenylamine or orcinol and by the phosphorus method are slight and within the limits of error of the method. Thus, the nucleic acid preparations which we obtained should be considered practically pure.

An important point in judging the value of the method is the determination of the loss of nucleic acids during fractionation. For this purpose, fraction 2 (residual tissue after extraction of NP) and fraction 6 (residual protein after removal of NA firmly bound to protein) were studied for their content of NA. Fraction 4 was tested for the presence of RNA (reaction with orcinol).

All these fractions were washed with 5% trichloracetic acid, then to each precipitate was added up to 4 ml of 5% trichloracetic acid and the samples with an air condenser stood 15 min on the water bath at 90°. Under these conditions, NA in the form of nucleotides passed into solution. In 5 min the samples were removed from the water bath, cooled, and centrifuged. The centrifugate was analyzed for content of phosphorus and NA.

Qualitative reaction with orcinol showed only traces of RNA in fraction 4.

The NA content in the trichloroacetic acid extract in fractions 2 and 6 was determined by the phosphorus method. The resulting data are given in Table 2. The amount of loss of nucleic acid from these fractions was 2-6% with respect to total amount of NA of the tissues. The greatest loss was found in fraction 2. The loss from fraction 6 was slight and did not exceed 1% with respect to the total amount of tissue NA. We should remark that the loss from fraction 2 could be decreased considerably by one washing of the residual tissue with 0.2% sodium hydroxide and combining the wash solution with fraction 3 (extract of NP and other proteins).

TABLE 1

Comparative Data on the Content of NA in Organs of the White Rat, Obtained by Two Different Methods (in mg phosphorus per g of fresh tissue•)

"Stable" DNA		"Labile" DNA		RNA	
by phosphorus method	diphenylamine reaction	by P method	diphenylamine reaction	by P method	orcinol reaction
Liver					
95	90	105	90	556	565
115	105	100	110	590	660
110	110	115	95	540	540
89	85	85	84	610	590
Average 102	97,5	101	92	574	588
Kidney					
125	110	125	120	220	210
104	100	95	90	237	215
115	120	85	75	250	260
90	100	110	115	240	220
Average 108	107	104	101	237	228
Spleen					
250	230	420	370	340	320
210	205	400	410	315	325
240	220	460	425	350	330
288	280	410	400	346	325
Average 247	234	422	401	338	325
Small intestine					
205	205	270	260	440	420
180	165	248	235	450	435
220	190	290	310	407	420
213	205	260	240	470	470
Average 204	191	267	281	442	436

•In all we made 31 studies. In the table we give figures from 4 experiments with maximum and minimum values.

TABLE 2

Content of NA Phosphorus in Fraction 2 (Residue of Tissues after Extraction of NP) and Fraction 6 (Protein Residue after Removal of Stable NA) (in mg per g of fresh tissue)

Fraction 2	Fraction 6	Total amount of loss	% loss with respect to total NA of tissue	Fraction 2	Fraction 6	Total amount of loss	% loss with respect to total NA of tissue
Liver				**Spleen**			
25	7	32	4	20	8	28	3
22	5	27	3	15	8	23	2
Kidney				**Small intestine**			
26	4	30	6	38	6	44	4
20	5	25	5	34	9	43	4

In connection with the question of the loss during fractionation it is important to emphasize that all the operations beginning with removal of the organs from the dead animal and up to placing the sample in the thermostat for hydrolysis, and also after hydrolysis, should be carried out in the cold (ice bath, temperature of working space not above 15°). Under these conditions, the data obtained by the method described above are quantitative.

DISCUSSION OF RESULTS

As a result of seeking a quantitative method for the separation of "stable" and labile compounds with protein and nucleic acids, it was found that part of the NA is precipitated from the alkaline extract of the tissues in the presence of sodium chloride by acetic acid, while another part of the NA remains in solution. It was assumed that the precipitated part of the nucleic acid was "stably" bound to the protein in the NP molecule, and the NA which remained in solution came from the "labile" NP which was split under these conditions. Experiments with repeated fractionation in great degree confirmed our assumptions.

We obtained data on the quantitative ratio of "stable" and labile compounds of protein and nucleic acids of liver, kidney, spleen, and small intestine of white rats. The greatest amount of "stable" NA was found in the spleen and small intestine, and less in liver and kidney. It was shown that labile NA contains both RNA and DNA, while "stable" contains only DNA. The purity of the preparations of NA was checked by two different methods: reaction for carbohydrates and for phosphorus. The results showed that NA preparations were practically pure. Tests of the probable loss of NA during fractionation gave reason to believe that the method used was quantitative.

In this work we again wish to call attention to the fact that in the fractionation of nucleic acids it is important to consider their property of forming different types of complex compounds with proteins.

SUMMARY

We have worked out a method for separating "stable" and labile compounds of proteins and nucleic acids in animal tissues which gives quantitative yields of practically pure preparations.

In all the organs studied the labile bond between NA and protein is present in greater amount than the "stable" bond. The ratio between the different types of nucleic acids varies with the tissue studied.

Received November 19, 1957

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THE SYNTHESIS OF ALANINE FROM PYRUVIC ACID AND AMMONIA BY A PURIFIED ENZYME PREPARATION FROM THE MITOCHONDRIA OF RAT LIVER

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The question of the mechanism of the synthesis of amino acids from ketoacids and ammonium salts has been a source of lively discussion in the biochemical literature for 20 years.

Soon after the discovery in 1937 of the Braunshtein and Kritsman process of transamination, Braunshtein [1, 2], von Euler [3], and Virtanen [4] expressed the opinion that in animal tissues direct amination was possible only for ketoglutaric acid (catalyzed by glutamic dehydrase). In all the other ketoacids (pyruvic, oxaloacetic, etc.) the formation of the corresponding amino acid could occur, in the opinion of these investigators, only indirectly, by first forming ketoglutaric acid from these acids in the Krebs cycle and aminating it, and then forming the corresponding amino acids by transamination from glutamic acid with excess added original ketoacids. To establish this theory, Braunshtein and his co-workers have in recent years carried out many studies of the relation between the process of amino acid synthesis from different ketoacids and ammonia and transamination reactions. It was established in these studies [5-8] that all the factors which prevent formation of ketoglutaric acid from pyruvic acid and the other ketoacids, and also all the enzyme poisons which prevent the transamination of glutamic acid, in corresponding degree also prevent the synthesis of amino acids from these ketoacids. On the basis of these results, Braunshtein in his general work published in 1957 stated categorically that in mammals the chief organs of nitrogen metabolism, the kidney and liver, apparently do not contain active enzyme systems for direct synthesis and direct oxidative deamination of any natural amino acid except glutamic acid [7]. It is important to say, however, that in respect to the data of these investigations, he gives only indirect evidence, not direct, that the synthesis of amino acids from pyruvic acid and other ketoacids occurs by transamination. Up to now nothing has been known about the nature and properties of the enzyme systems which can catalyze direct amination of pyruvic and other ketoacids besides ketoglutaric acid, and so we cannot exclude the possibility that factors which prevent the process of transamination or the formation from pyruvic acid of ketoglutaric acid may not also prevent the process of direct amination.

A basic objection by the supporters of the idea of direct amination [9-12] against the theory of formation of amino acids from pyruvic and other ketoacids only by transamination is the fact that when a liver preparation is incubated with pyruvic acid and ammonia, those amino acids are synthesized which correspond to the starting ketoacids. Thus, the amount of glutamic acid fromed in the synthesis from pyruvic acid is much less than the amount of alanine synthesized. If a liver slice is treated simultaneously with pyruvic and ketoglutaric acids, as Kaplanskii and Berezovskaia showed [10], the corresponding amounts of alanine and glutamic acid are formed, indicating that these two processes are independent. This evidence for the presence in the liver of an enzyme system which can aminate pyruvic acid directly is, however, as Braunshtein showed [7], also only indirect.

Thus, the question of the mechanism of amino acid synthesis from ketoacids, except ketoglutaric acid, remains open, and new, direct evidence is necessary for its solution. One such piece of evidence would be the

separation from liver or kidney of an active enzyme system which could catalyze the synthesis of alanine from pyruvic acid but could not catalyze the transamination between pyruvic and glutamic acids. The isolation of such a system is the problem of our work.

EXPERIMENTAL SETUP AND METHOD OF INVESTIGATION

In choosing a method for separation of an active enzyme preparation which would catalyze the amination of pyruvic acid, we started from data obtained earlier by one of us [13] which showed that this process can occur in liver mitochondria in which this process is localized [14] when they have been disintegrated by repeated freezing and thawing. The methods of isolating the mitochondria, obtaining the extracts from them, and determining the amounts of amino acid formed were the same as in our previous work [13, 14]. It is important only to say that the fractionation of the liver tissue took place in this work in buffered sucrose which contained 0.85% KCl. The extracts obtained from the disintegrated mitochondria contained about 1% protein; they were then submitted to electrophoresis on starch. Electrophoresis was carried out in 0.05 M K-phosphate buffer (pH 7.4). Electrophoresis lasted 16-18 hours with a potential gradient ~6 v/cm and 20 m a. At the end of the electrophoresis the starch was cut into 13 separate blocks, each 2 cm wide, and the protein from each strip was extracted with 6 ml of cooled K-phosphate buffer. The extracts were centrifuged or filtered in the cold and then the protein in them was determined by the Lowry method. We determined the intensity of formation of amino acid in separate portions of the extracts after incubation with pyruvic acid and ammonium carbonate [14], and also the activity of the two transaminases, glutamic–aspartic and glutamic–alanine. The activity of these enzymes was determined by the increase in glutamic acid after incubation of the extracts with ketoglutaric and aspartic acid, or ketoglutaric acid and alanine. A quantity of substrate of 0.2 ml of 0.1 M solution per sample was incubated 1 hour at 37°. The amount of extract added to the sample 0.7 ml, total volume of sample was 1.5 ml; the required volume was reached with phosphate buffer, pH 7.4. At the end of the incubation, the protein was precipitated with trichloroacetic acid (TCA) which was removed from the filtrate by ether extraction; the glutamic acid formed was determined by the method of Giri [15] after separation of the amino acids by paper chromatography (mobile phase – phenol saturated with water). As controls we used samples in which the extract was incubated in the presence of ketoglutaric acid but without adding aspartic acid or alanine.

RESULTS OF THE INVESTIGATION

In Fig. 1 we give the curves of distribution of total protein and intensity of synthesis of amino acids in extracts from separate fractions obtained on starch in a characteristic experiment.

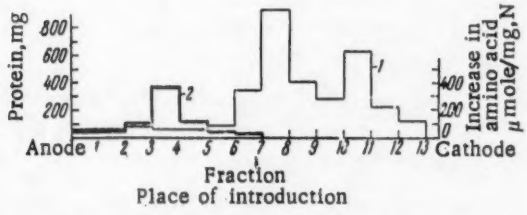

Fig. 1. Separation of total protein and enzyme which catalyzes synthesis of amino acid from pyruvic acid and NH_3 in starch electrophoresis of protein extract from disintegrated mitochondria: 1) total protein content, 2) activity expressed by increase of amino acid in the extracts.

As this figure shows, considerable synthesis of amino acid (expressed in μ mole per mg N) from pyruvic acid occurs only in those parts of the extract which had been obtained from the starch sections near the anode. The most active extract was obtained from the fourth starch section which was located 6-8 cm from the anode. These results, therefore, fully confirm those obtained in our previous work [13].

The intensity of the transamination process (expressed in μ mole increase in glutamic acid per mg N) in the extracts obtained from the various starch blocks is given in the curves in Fig. 2. When we compare the curves of synthesis and transamination it is clear that in all the extracts in which intensive synthesis occurs, transamination is completely absent. On the other hand, in the extracts in which there is considerable transamination, we cannot find any synthesis of amino acids from pyruvic acid. This is also apparent from the chromatograms in Fig. 3. In this figure, the numbers 1, 2, 3, etc. denote extracts obtained from separate starch blocks, beginning from the anode end. In samples denoted by the letter a we determined the activity of glutamic acid-aspartic acid transaminase, and in samples denoted by b, the activity of glutamic acid-alanine transaminase. The upper spots, as the legend shows, correspond to aspartic acid, the middle ones

to glutamic acid, and the lower ones to alanine. The chromatogram shows that weak spots of glutamic acid in samples where the activity of glutamic acid – aspartic acid transaminase was studied occur only in the seventh sample, that is, the sample 14 cm away from the anode. The greatest intensity of this transaminase system is found in samples 10-11, that is, in extracts obtained from starch blocks 20-22 cm distant from the anode. As to the intensity of the glutamic acid–alanine system, clear spots of glutamic acid appear only in sample 10, and traces of glutamic acid are observed in samples 9 and 11.

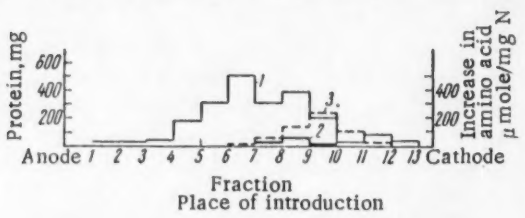

Fig. 2. Separation of total protein and transaminases; glutamic acid-aspartic acid and glutamic acid – alanine in electrophoresis on starch of protein extracts of disintegrated mitochondria: 1) total protein content. Activity of transaminases by increase in glutamic acid; 2) glutamic acid–alanine; 3) glutamic acid–aspartic acid.

Thus, the results show that in those extracts in which synthesis from pyruvic acid occurs, transamination processes are completely absent, whether between ketoglutaric and aspartic acid or ketoglutaric acid and alanine.

To answer the question of just how amino acid is formed in the incubation of extracts with pyruvic acid we determined the amino acids formed by a series of chromatographic experiments.

In Fig. 4 we give the results of the experiments carried out with extract 4. In experimental sample a we see plainly only a spot which corresponds to alanine. In the control sample b, which was incubated under the same conditions but without pyruvic acid and ammonium salt, the alanine spot is very weak, and the same is true of the second control c, which contained all the needed substrates, but which was fixed by TCA immediately after adding the substrate.

In some cases it is possible to see on the chromatogram a very weak spot which corresponds to glutamic acid. The appearance of this spot is probably connected with the presence in the extract being studied of traces of ketoglutaric acid. The results of these experiments thus indisputably show that in the extracts which we studied and which contain no glutamic acid transaminase system, incubation with pyruvic acid and ammonium salt produces synthesis of alanine.

DISCUSSION OF RESULTS

The process which we have observed of synthesis of alanine from pyruvic acid and ammonium salt in purified preparations from liver mitochondria which do not contain transamination enzyme systems is positive evidence for the possibility of direct amination of pyruvic acid in liver tissue.

As we showed in our previous work [13] the activity of enzyme systems which catalyze direct amination of pyruvic acid, when purified by starch electrophoresis, is about 300 times as high as in liver homogenates, and it can be considered that further purification would raise still more the activity of these systems.

In considering the mechanism of amination of pyruvic acid by the isolated enzyme system, we come naturally to the question of the origin of the hydrogen required for this process. This question is the subject of our further study. We can at once express the opinion that in our purified system along with the enzyme which catalyzes the amination of pyruvic acid there is also an enzyme capable of oxidizing part of the pyruvic acid, or perhaps other substrates in the system, and liberating the hydrogen required for the amination of pyruvic acid. Such a possibility is indicated by the work of Gamble and Lehninger [16] who showed that in various fractions obtained from disintegrated mitochondria by the ultracentrifuge, an enzyme system was always present which could oxidize succinic acid, β-hydroxybutyric acid, and other substrates.

The presence of enzyme systems which catalyze direct amination of pyruvic acid in the absence of transamination enzymes, finally, does not deny the role of transamination in the formation of amino acid from pyruvic and other ketoacids; it only indicates the existence of two paths for this process. The results obtained by Braunshtein and his co-workers in the investigation of liver slices and homogenates [5, 6] show that in some cases the specific role of transamination in the formation of amino acids from ketoacids is very great.

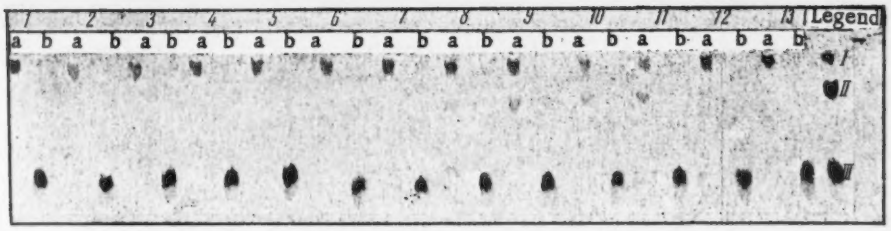

Fig. 3. Separation of glutamic acid–aspartic acid and glutamic acid–alanine transaminases in starch electrophoresis of protein extracts from disintegrated mitochondria. Substrates: a) α-ketoglutaric acid and aspartic acid; b) α-ketoglutaric acid and alanine. Spots (at 672 6 μg): I – aspartic acid; II – glutamic acid; III – alanine

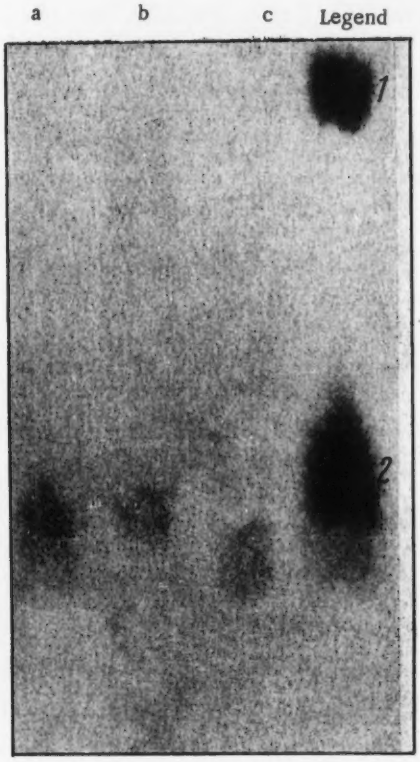

Fig. 4. Formation of alanine in incubation of the extract from the fourth (from the anode end) starch section after starch electrophoresis of extracts from disintegrated mitochondria. Substrates: a) Na pyruvate and $(NH_4)_2CO_3$; b) K-phosphate buffer; c) Na pyruvate and $(NH_4)_2CO_3$ without incubation; I – glutamic acid; II – alanine

However, the question of the role of direct and indirect amination in vivo is entirely unsettled.

It is important to say also that if the enzyme systems of direct amination are more labile than the transamination enzyme systems, and are partly destroyed during isolation, then in experiments in vitro the activity of the indirect amination systems will naturally predominate.

This question requires further study, but from the results of these investigations, we can say now that there is no possibility of denying the presence and value of direct amination of ketoacids in the synthesis of amino acids.

SUMMARY

Using starch electrophoresis of extracts of disintegrated mitochondria from rat livers we have been able to isolate an enzyme system which catalyzes the synthesis of amino acids from pyruvic acid and ammonium salts in the absence of glutamic acid–alanine and glutamic acid–aspartic acid transaminases.

By paper chromatography we have shown that the product of this synthesis is alanine.

The results give positive evidence of the presence in rat livers of enzyme systems which catalyze the direct amination of pyruvic acid.

Received December 2, 1957

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THE FUNCTIONAL STATE OF THE CENTRAL NERVOUS SYSTEM AND PHOSPHOPROTEIN METABOLISM OF THE BRAIN

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In studies on the biochemistry of the central nervous system in recent years much attention has been devoted to the investigation of the phosphoproteins (PP) of brain tissue.

It has been shown in experiments on brain sections that PP metabolism is closely connected with such vital processes as respiration, glycolysis, oxidation of phosphorylated compounds, and others [1-7].

The phosphoproteins are characterized by a high rate of renewal which is considerably greater than the metabolism of phospholipids or nucleic acids, and which approaches the renewal rate of high-energy phosphates [8-10]. These and other data give reason to suppose that PP are related to these protein structures of the brain which can be directly connected with the specific functional activities of the central nervous system. There is direct evidence in the literature for the existence of such a connection. Thus, Vladimirov [8] showed that with unconditioned or conditioned reflex stimulation of rats, the renewal rate of PP rose greatly. According to the data of Skvirskaia and Silich [11], prolonged narcotic sleep caused by a mixture of medinal and urethane decreased the introduction of P^{32} into PP fractions of the rat brain. Chetverikov [12] showed that at low barometric pressures the rate of renewal of PP in rat brain was decreased. Very recently, Heald [13] has shown that with short electrical stimulation of the cerebral cortex of guinea pigs, the introduction of P^{32} is hastened only in the PP fraction.

The problem of our work is to investigate the renewal rate of brain phosphoproteins in different functional states of the central nervous system.

METHOD OF INVESTIGATION

The experiments were carried out on rabbits and rats. The animal was injected with a solution of $Na_2HP^{32}O_4$ with a dose of 100-190 thousand counts per min in 1 g of substance for rabbits and 500-600 thousand counts per min per g for rats. After 2 hours the animals were killed and the brain removed. In some of the experiments the rabbit head was frozen in situ by the method used in our laboratory [14]. Determination of the amount and rate of renewal of PP was carried out according to the method of Vladimirov and co-workers [15] as follows. The brain was ground in water (1:4) and an equal volume of 10% trichloroacetic acid (TCA) was added. After 18-20 hours it was centrifuged and aliquot parts of the centrifugate were taken for determination of the amount and radioactivity of the inorganic phosphate (IP). The residue was washed 8 times in the centrifuge with 5% TCA, and the lipids were removed by successive treatment with acetone, a mixture of alcohol and ether (1 : 3) with heating, a mixture of chloroform and methanol (1:1) with heating, and ether. After removing the traces of ether we hydrolyzed the protein residue in N NaOH at 37° for 22 hours. It was neutralized with 10 N H_2SO_4. TCA was added to a final concentration of 5%, it was filtered and the IP precipitated according to Delory. The phosphate precipitate was washed and dissolved in 0.1 N HCl. In this solution, just as in the first TCA centrifugate, which contained IP, we determined the amount of phosphate by the Fiske-Subbarow method. After photometric determination, the colored solution was extracted with isobutanol. We placed on a filter paper disk, diameter 16 mm, four successive applications of 0.2 ml of isobutanol extract (each time with drying), and measured the radioactivity with a counter MTS-17 and an apparatus of type B. The radioactivity

of each of the two studied phosphate fractions (IP and PP) was expressed as the specific activity (SA), that is, we calculated the number of counts/min per g of phosphorus. To characterize the rate of renewal of PP we calculated the relative specific activity (RSA), the ratio of SA of phosphoproteins to SA of inorganic phosphate, expressed in percent. Each result was the average of two parallel determinations.

In our study we confirmed the conclusions in the work of Vladimirov and co-workers [15] that in the precipitate of inorganic phosphate obtained after hydrolysis of PP there is a considerable amount of organic phosphorus compounds which have a high radioactivity. We showed that for extraction of the colored phosphomolybdenum complex we could use not only isobutanol, but also isoamyl alcohol. In the case of isobutyl alcohol, our data show that removal of radioactive phosphorus reached 97%, and with isoamyl alcohol, 95.3%. We also showed that during removal of the phosphoprotein fraction it was not necessary to remove the nucleic acids. According to our results, the content of PP phosphorus of rat brain averaged 1.27 mg% when nucleic acids were not removed and 1.39 mg% when they were.

TABLE 1

Degree of Contamination of PP Phosphorus by the Radioactivity of Inorganic Phosphate

Experimental conditions	Inorganic phosphorus		PP phosphorus		RSA in %
	in μg/g of wet tissue	SA	in μg/g of wet tissue	SA	
Introduced 40,000 counts per min per g of brain; centrifuged after 10 min	432	103 000	25.0	730	0.7
	422	96 000	27.6	—	—
	381	110 000	24.0	980	0.9
	365	120 000	22.2	1060	0.9
	410	115 000	25.6	870	0.8
	400	115 000	26.4	1750	1.1
Average	402	110 000	26.4	1080	0,9
Introduced 11,000 counts per min per g of brain; centrifuged after 20 hours	457	18 800	25.2	1040	5.4
	489	14 900	26.2	1200	8.0
	336	20 800	23.4	880	4.2
	336	20 200	22.8	910	4,5
	387	18 700	26.0	1530	8.2
	374	18 500	24.0	1980	10.7
Average	396	18 650	24.7	1250	6.8

Since the content of PP phosphorus in brain tissue is very small compared to the total amount of phosphorus in the brain, it is very probable that during the complex fractionation of brain tissue used in the determination of PP, there could have been some contamination of this fraction by inorganic radioactive phosphates with high specific activity from blood, cerebrospinal fluid, or brain tissue. In order to estimate the possibility of such contamination, we ran a special series of experiments in which the ground up brain was treated with radioactive phosphate at 11,000-40,000 counts/min per g of tissue, and the protein was slowly precipitated by TCA. Part of the samples were worked up after 10 min and part remained in TCA for 18-20 hours, according to our method. In this case all the radioactivity observed in the phosphoprotein fraction came from contamination, since renewal of phosphoprotein could not occur here.

The results of these experiments are given in Table 1 and show that if precipitation of proteins is limited to a short time and centrifuging begins 10 min after adding TCA, and the brain does not remain in TCA for a day, as we had done, then the PP fraction shows practically no radioactivity. With longer standing of the brain suspension with TCA we noted in the PP fraction measurable radioactive contamination which in units of relative specific activity reached 6.8%. Hence the true rate of renewal of PP is lower than the value which we determined.

RESULTS OF THE INVESTIGAION

For the first series of experiments we determined whether freezing the brain affected the content and rate of renewal of PP. The experimental procedure was as follows.

Rabbits were injected with radioactive phosphate and after 2 hours the brain was frozen in situ. The frozen brain was removed, one half hemisphere was ground up in the frozen state with liquid oxygen and a weight of 1 g was taken in a measured amount of TCA. The other half remained at room temperature until completely thawed (30-60 min) and then it was treated as described.

TABLE 2

Content and Rate of Renewal of Phosphoproteins in Frozen and Thawed Brain

Experimental conditions	Inorganic phosphorus		PP phosphorus		RSA in %
	in μg/g wet tissue	SA	in μg/g wet tissue	SA	
Frozen	148	12 200	29.1	4020	33.0
Thawed	291	8 450	20.8	3120	36.7
Frozen	159	12 900	29.5	3900	30.3
Thawed	274	9 150	15.6	2900	31.6
Frozen	204	15 300	33.4	5340	34,8
Thawed	378	16 400	18.8	3620	22.0
Frozen	216	15 400	31.2	4850	31.4
Thawed	302	19 600	23.2	3880	19.8
Frozen	200	12 200	31.1	4980	40,7
Thawed	332	12 100	20.4	4170	34.4
Frozen	246	11 000	31.2	4000	36,4
Thawed	354	11 000	23.2	3900	35.4
Frozen	203	13 400	29.9	5400	40.0
Thawed	342	8 900	22.6	3050	34,3
Frozen	246	10 720	29.8	4300	40.0
Thawed	458	8 000	15.7	2800	35.0
Av. of frozen	203	12 900	30,6±0,49	4600	35.8±1.45
Av of thawed	342	11 700	20.0±1.18 $p<0.001$	3440	31.1±2,29 $p=0.11$

Table 2, in which we give the results of these experiments, shows that in all cases of thawing of the brain there was an increase in the amount of inorganic phosphate which was easily explained by splitting of labile phosphorus compounds (adenosine triphosphate, creatine phosphate), and a slight but definite ($p < 0.001$) decrease in content of PP phosphorus. This decrease indicates that brain PP is definitely labile. It is interesting that in spite of the decrease in absolute amount of PP, the rate of renewal, expressed by the RSA is almost unchanged ($p = 0.11$). It follows from this that part of the PP which splits in thawing does not actually differ in rate of renewal from the rest of the PP.

It is known that thawing of frozen tissue can cause damage, and so as a control we ran a series of experiments in which, after we had introduced the radioactive phosphorus, the rabbit brain was quickly removed without freezing and half a hemisphere was slowly immersed in liquid air while the rest remained at room temperature for 30-60 min.

From Table 3, which shows the results of these experiments, it is evident that removal of the brain after freezing does not affect the level of PP. At the same time, the amount of PP in the experiments of Table 3 is somewhat lower than the content in brain frozen in situ (Table 2), ($p < 0.001$). From this it follows that decrease in the amount of PP occurs very quickly after decapitation of the rabbit, and further keeping of brain tissue for 30-60 min at room temperature does not change the level. However, the amount of PP in fresh brain tissue is actually higher ($p < 0.001$) than in tissues thawed after freezing. This is an indication that the injurious action of freezing and thawing is reflected in the level of PP.

It is important to observe that the values of RSA in Table 3 are higher than those in Table 2. This is because in the experiments of Table 3 the rabbit was decapitated, which led to less blood in the brain tissue, so that contamination of the brain with radioactive IP from the blood is less and the SA of inorganic phosphate is correspondingly lower.

TABLE 3

Content of Phosphoproteins in Fresh and Frozen Rabbit Brain

Experimental conditions	Inorganic phosphorus		PP phosphorus		RSA in %
	in μg/g wet tissue	SA	in μg/g wet tissue	SA	
Frozen	464	6950	23.8	2840	40.9
Fresh	512	6210	23.8	2860	46.0
Frozen	492	6300	22.5	3060	48.5
Fresh	520	6630	23.8	2970	44.8
Frozen	392	9800	24.2	3750	38.2
Fresh	584	8640	26.2	3740	43.3
Frozen	396	7600	24.8	3390	44.6
Fresh	560	8100	27.6	—	—
Average Frozen	436	7660	23.8	3260	43,0
Average Fresh	544	7400	25.3	3190	44.7

Since treatment of fresh brain tissue without freezing in situ permits freeing it from soft brain membranes and hence from extra amounts of blood, and this method of treatment has no effect on rate of renewal of PP, in all the following experiments we did not freeze the brain in situ.

Parallel with the biochemical study we also made an objective evaluation of the functional state of the animal by recording the brain waves, blood pressure, and respiration.

Under light ether narcosis we performed a tracheotomy and exposed the femoral artery. Steel needle electrodes were placed at symmetrical points on the cranium. The brain waves were conducted off by a bipolar setup, with a distance between the poles of 6-8 mm. Some of the animals were paralyzed by intravenous injection of diplacin (2 mg / kg) and given artificial respiration. Recording of blood pressure was carried out in the blood of the femoral artery. The electroencephalogram was recorded with a train oscillograph.

In Table 4 we give the data for rate of renewal of brain PP of rabbits in a state of relative rest.

As Table 4 shows, artificial respiration does not affect the rate of renewal of brain PP.

Figure 1 gives a typical kymogram and electroencephalogram (EEG) from the experiments of this series. The injection of radioactive phosphorus does not change the blood pressure or bioelectric activity of the cortex of the large brain hemispheres during the experimental period.

In the next series of experiments, whose results are shown in Table 5, we studied renewal of PP in a two hours cramp produced by corazole*. Corazole was injected intravenously in repeated doses (10-20 mg/kg), 4-6 times to assure continuous cramping activity during the entire experimental period.

As Table 5 shows, corazole cramp did not cause any change in content or rate of renewal of PP. The observed changes in blood pressure and EEG were characteristic for corazole (Fig. 2).

For comparison, the rate of renewal of PP was studied not under conditions of stimulation, but with inhibition of the central nervous system. As the inhibiting agent we used diethylaminoethyl benzoate (ester 22) which, according to the data of Krylov [16] and Selivanova [17] produces an inhibitory process in the cortex of the large hemispheres of the brain. Ester 22 was injected in doses of 2 mg / kg intramuscularly (Fig. 3).

The results of this experiment, shown in Table 5, demonstrate that with inhibition of the central nervous system the rate of renewal of PP is not changed. The difference between the value of RSA with inhibition of the central nervous system (46.0%) and in cramp (41.2%) are insignificant ($p = 0.19$).

* Pentamethylenetetrazol.

TABLE 4

Rate of Renewal of Brain Phosphoprotein in Control Rabbits

Experimental conditions	Inorganic phosphorus		PP phosphorus		RSA in %
	in μg/g wet tissue	SA	in μg/g wet tissue	SA	
Without artificial respiration	468	13 100	15.2	5450	41.5
The same	602	15 900	19.0	7500	47.0
Artificial respiration	467	7 860	23.0	3300	42.0
The same	550	8 745	28.5	3650	41.7
Without artificial respiration	425	14 600	22.0	6600	45.2
The same	596	12 770	24.0	4990	39.0
"	416	9 960	13.3	5070	50.8
Artificial respiration	467	10 550	27.8	3950	37.5
	461	10 600	28.2	5570	48.7
Average	—	—	—	—	43.7±1.49

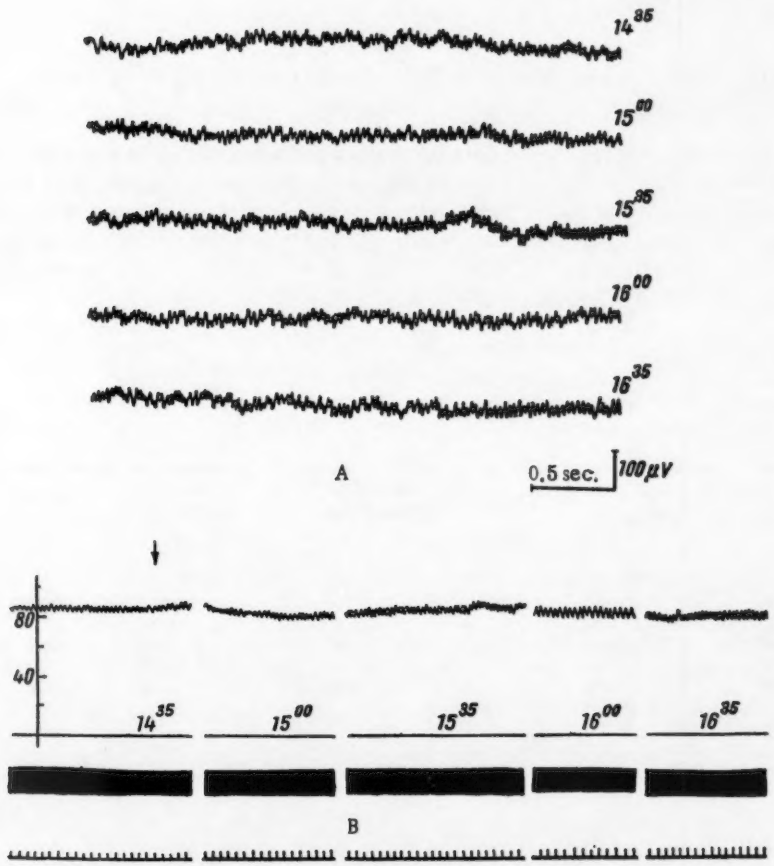

Fig. 1. Electroencephalogram (A) and kymogram (B) of control rabbits. B – from above down: blood pressure, zero line of blood pressure, respiration, time marker 0.5 sec, →injection of $Na_2HP^{32}O_4$.

TABLE 5

Rate Of Renewal of Rabbit Brain Phosphoprotein in Cramp Produced by Corazole and with Injection of Ester 22

Inorganic phosphorus		PP phosphorus		RSA in %	Experimental conditions	SA inorganic phosphorus	SA of PP phosphorus	RSA in %
in μg/g wet tissue	SA	in μg/g wet tissue	SA					
Corazole					Ester 22			
488	11 600	19.9	5660	48.8	Without artificial respiration	13 400	6050	45.0
394	18 200	26.0	6170	33.8	The same	5 040	2120	42.1
491	12 000	23.2	6340	52.6	Artificial respiration	6 620	2540	38.4
464	16 050	27.4	6220	38.8	Without artificial respiration	8 250	4240	51.4
450	16 400	28.8	5770	35.2	The same	4 580	2210	48.2
522	24 200	25.1	9300	38.4	Artificial respiration	8 050	4110	51.1
					Without artificial respiration	9 140	4370	48.0
					The same	8 870	3780	42.6
		Average		41.2±3.12				46.0±1.64

Since most of the literature data on brain PP were obtained with rats, we decided to carry out some of our experiments with these animals.

As has been shown earlier [18], corazole does not produce prolonged cramps in rats, and so we used intramuscular injections of phosphacol* with a dose of 0.25 mg/kg. Under these conditions, after 6-10 min from the time of injection in rats there occurred cramp which lasted for several hours with short interruptions. The animals were killed 2 to 24 hours after injecting phosphacol. In all these experiments radioactive phosphate was injected intramuscularly 2 hours before the animal was killed. Control rats were injected intramuscularly with physiological saline instead of phosphacol.

TABLE 6

Content and Renewal Rate of Brain PP of Rats in Normals and Under The Influence of Phosphacol

SA of inorganic phosphorus	SA of phosphoproteins	RSA in %	SA of inorganic phosphorus	SA of phosphoproteins	RSA in %
Normal			2-hour Cramps		
43 000	15 000	34.9	61 000	24 000	39.4
47 000	13 000	27.7	43 000	15 000	34.4
54 000	19 000	35.2	57 000	19 000	33.4
39 000	16 000	41.0	25 000	8 000	32.0
43 000	15 000	34.9	19 000	7 000	36.8
20 000	4 400	27.0			
16 000	6 000	37.4			
Average		34.0	Average		35.2

For the analyses in Table 6 we combined the brains of four rats; the results show that two-hour cramp caused by phosphacol did not affect the intensity of uptake of P^{32} in the PP fraction. Analogous data were obtained for cramp lasting 24 hours.

* diethyl-p-nitrophenyl phosphate.

Thus, neither stimulation caused by substances with differing types of activity (corazole, phosphacol), nor inhibition produced by ester 22 affects the rate of uptake of P^{32} by the PP fraction of the brain.

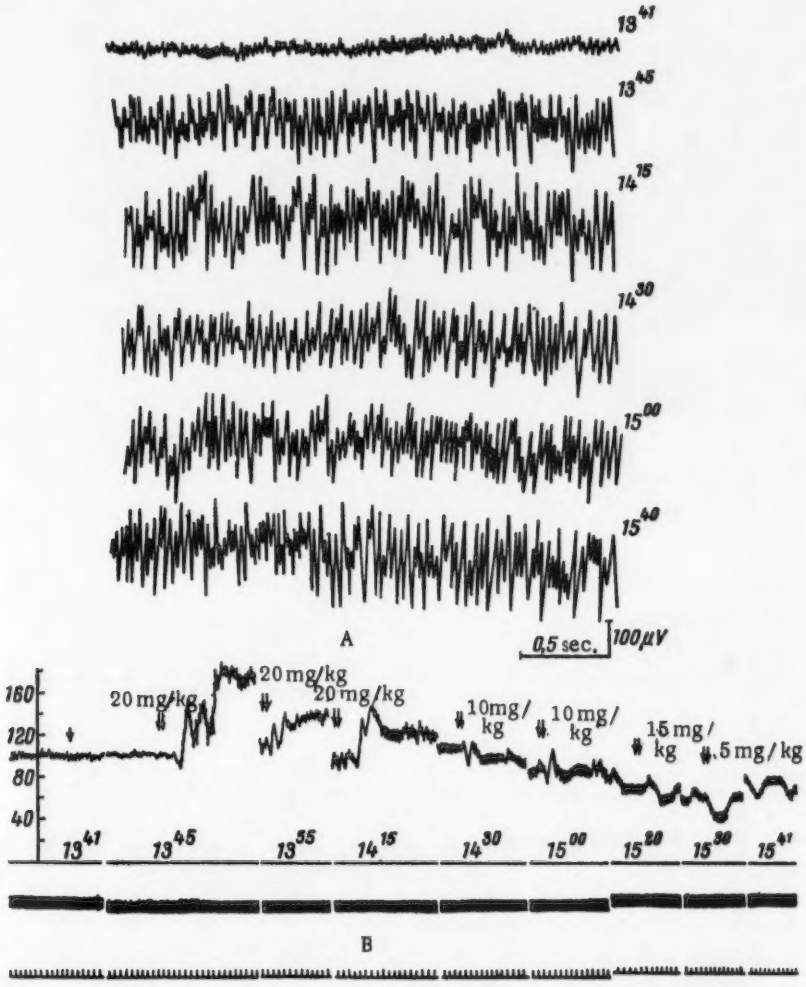

Fig. 2. Electroencephalogram (A) and kymogram (B) of rabbits poisoned with corazole. Designations the same as in Fig. 1; ↓ ↓ injection of corazole.

The results of our investigation of stimulating agents on the brain do not agree with the data of Vladimirov cited above [8], which showed that conditioned reflex activity increased renewal of phosphoproteins of brain tissue. The reasons for this difference are not clear to us, but it may be assumed that the experiments of Vladimirov in this investigation [8] were carried out without taking account of the improvements which he and his co-workers later introduced [10]. As to the experiments on inhibition of the central nervous system, the literature available to us showed only one study of the rate of renewal of brain phosphoproteins under these conditions.

Skvirskaia and Silich [11] studied the uptake of P^{32} in PP (and other phosphorus-containing compounds) in rat brains of normals and during prolonged narcotic sleep. The animals were killed 2 and 4 hours after injecting the P^{32}. According to their results, the greatest difference for PP was found after 2 hours. Under these conditions the RSA for PP phosphorus in the normals was 27.7% and during narcotic sleep, 22.4%, that is, during inhibition, renewal of brain PP was 19.2% lower than in a state of relative rest. From this the authors

concluded that during prolonged narcotic sleep the rate of uptake of P^{32} in brain PP was decreased.

We submitted the numerical data of Skvirskaia and Silich [11] to statistical treatment by the method of Student-Fisher [19]. This showed that the differences between the series of experiments were not significant ($p = 0.33$), that is, narcotic sleep did not affect the renewal of brain PP.

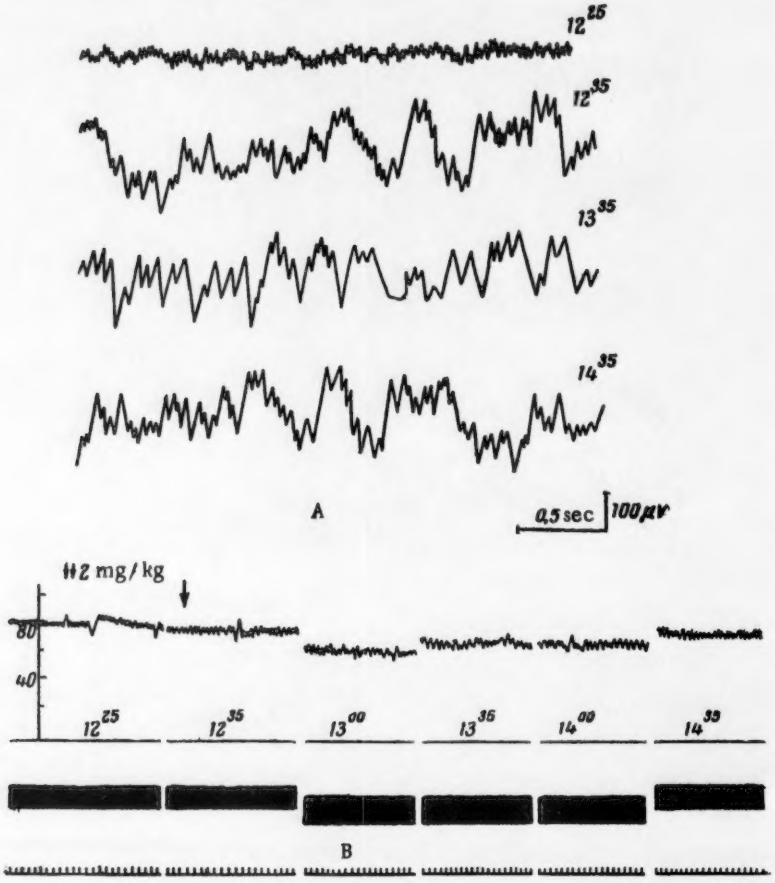

Fig. 3. Electroencephalogram (A) and kymogram (B) of rabbits injected with diethylaminoethyl benzoate. Designations the same as in Fig. 1; ↓↓ injection of diethylaminoethyl benzoate.

Thus the actual data of Skvirskaia and Silich agree with the results of our experiments, carried out under different conditions and with different animals.

SUMMARY

The phosphorus content of phosphoproteins (PP) in the brain which has been frozen in situ is higher than in thawed or freshly removed brain which has not been frozen. Thawing of a frozen brain does not change the value of RSA for PP phosphorus.

Cramps caused by corazole (in rabbits) or phosphacol (in rats) affect neither the content nor the rate of renewal of brain PP. Inhibition of the central nervous system in rabbits produced by injection of diethylaminoethyl benzoate is not accompanied by change in renewal rate of brain PP.

Received December 6, 1957

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DEAMINATION OF L-AMINO ACIDS AND GLYCINE IN LIVER AND KIDNEY TISSUE OF BIRDS

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Until recently no one has studied the question of deamination of L-amino acids in the tissues of birds. The literature contains only a few indications of the amount of deamination, chiefly of racemic mixtures of amino acids or their unnatural isomers by D-amino acid oxidase [1-5].

In studies of the oxidation of amino acids the deamination is often determined by absorption of O_2 without reckoning the formation of NH_3. It is known that the oxidation of some amino acids can proceed without simultaneous splitting out of NH_3, and therefore determination of the value of Q_{O_2} does not always reflect the true level of deamination of amino acids.

The work of Boulanger and Osteux, published in 1949-56 [6-10], deserves attention among the studies on deamination of amino acids in bird liver and kidney. These authors studied the deamination of L-diamino acids, aromatic and some other amino acids by liver homogenates and by acetone extracts of turkey livers. They first showed the presence in turkey livers of an active specific L-amino acid oxidase.

Our study is the first systematic attempt to study the deamination of the principal natural amino acids in bird tissues. Our problem is to show the intensity of the deaminative splitting of L-amino acids in bird liver and kidney, as representatives of the uric acid type of nitrogen metabolism.

Below we give experimental data on the deamination of L-amino acids in liver and kidney slices and homogenates and also in extracts of acetone-treated bird livers. Our work was carried out during 1955-1956.*

EXPERIMENTAL PART

As objects of investigation we used liver and kidney tissue from pigeons, 20-90-day-old chickens, turkey poults and adult turkey hens. The amount of deamination of amino acids was estimated by increase in ammonia nitrogen found in samples during aerobic incubation in the presence of amino acids. Ammonia nitrogen was determined spectrophotometrically (in a Spekker absorptiometer) after isothermal distillation of NH_3 according to Conway and nesslerization.

Deamination of Amino Acids by Tissue Slices

In this series of experiments we studied the deamination of 13 L-amino acids, glycine, and some D- and DL-amino acids in slices of liver and kidney from pigeons, chickens, and turkeys.

The samples which contained 100 μmole of neutralized amino acid in 2 ml volume and 100 mg of tissue in Krebs-Ringer-bicarbonate salt solution, pH 7.4 were incubated for 2 hours under aerobic conditions in a

* In 1957 Struck and Sizer [11] described the oxidative deamination of a series of natural amino acids by enzyme preparations from the liver of chickens. The authors showed that the enzyme activity was centered in the microsome fraction of the cell granules.

shaker at 38°. One sample was the control; it contained no amino acid. After incubation the protein was precipitated by addition to the sample of 1 ml of 20% CCl_3COOH. Part of the trichloroacetic acid filtrate was hydrolyzed for 15 min on a boiling water bath to liberate NH_3 from the amino group of glutamine, the ammonia was distilled into a receiver and determined with the Nessler reagent.

Separate experiments were run which showed that after hydrolysis under such conditions (final concentration of CCl_3COOH 5-6.6%) there was full liberation of ammonia from the amino group of glutamine which formed in the liver and kidney during incubation. The amount of "endogenous" ammonia formed in the tissue samples which were incubated without addition of amino acids was usually 20 to 55 μmole per g of tissue in the liver, and 35 to 80 μmole per g in the kidney.

TABLE 1

Amount of Deamination of Amino Acids in Slices of Bird Liver and Kidney (increase in $N\text{-}NH_3$ in μmole per g of tissue in 2 hours)

Amino acid	Pigeon		Kidney		Kidney	
	liver	kidney	liver	kidney	liver	kidney
L-Glutamic acid	23	40	13	31	19	24
L-Aspartic acid	33	60	19	70	32	55
L-Alanine	33	59	28	69	19	114
L-Valine	3	14	14	18	15	4
L-Leucine	18	2	0	44	26	25
L-Isoleucine	14	15	13	40	11	12
L-Phenylalanine	21	3	21	31	35	10
L-Tyrosine	1	0	5	15	29	13
L-Tryptophan	8	7	4	7	66	30
L-Lysine	8	19	11	3	54	4
L-Arginine	43	29	44	32	83	25
L-Ornithine*	—	—	—	—	37	—
L-Methionine*	—	—	—	—	51	—
Glycine	6	12	3	0	20	10
D-Alanine	23	—	17	—	—	—
DL-Alinine	47	—	37	—	—	—
D-Phenylalanine	127	222	55	—	—	—
DL-Phenylalanine	11	—	25	—	—	—
DL-Lysine	—	—	—	—	24	—
D-Tryptophan	—	—	—	—	10	—
DL-Tryptophan	—	—	—	—	24	—

*In samples with DL-amino acids and sodium benzoate (final concentration $M \cdot 10^{-2}$).

Table 1 shows the amount of deamination of different amino acids in liver and kidney slices from the birds which we studied. The amount of deamination is expressed in increase in ammonia nitrogen in μmole per g of wet tissue with 2-hour incubation. The values in Table 1 are averages of several experiments.

The results shown in Table 1 demonstrate that deamination of the series of L-amino acids occurs in bird liver and kidney tissue with a sufficiently great intensity.

Deamination of L-glutamic acid, L-aspartic acid, and L-alanine in the tissue slices of the studied birds occurs with greater intensity than does deamination of these amino acids in mammalian tissue [2,12-14]. These amino acids are particularly well deaminated in bird kidney slices. Of the other L-monoamino acids (except L-alanine), L-valine, L-leucine, and L-isoleucine are deaminated with marked speed. Deamination of leucine and isoleucine in chicken and turkey kidneys sometimes reaches a considerable size (44 and 26 μmole per g of tissue). In this series of experiments we also observed deamination of the aromatic amino acids: L-phenylalanine and L-tyrosine. L-Phenylalanine was actively deaminated in chicken kidney and in the livers of all the birds studied. The highest value for deamination of L-phenylalanine was found in turkey liver. Deamination of L-tyrosine was sometimes characterized by a rather low value, apparently because of the poor solubility of this amino acid.

We have observed deamination of L-tryptophan for the first time; it proceeds intensely in turkey liver tissue and also in kidney slices from this bird. Deamination of L-tryptophan in our experiments was accompanied by the appearance of a yellow or red color in the incubation mixture. The intensity of the color paralleled the intensity of deamination of the tryptophan. An analogous effect was observed by Sourkes and Townsend in a study of oxidation of L-tryptophan by cobra venom [15].

In liver and kidney slices of the birds which we studied, as we showed, L-diamino acids were actively deaminated. We found a high value for the deamination of L-lysine in turkey liver slices; L-arginine was deaminated with the same high speed in liver and kidney slices of all the birds.

We also found deamination of L-ornithine and L-methionine in turkey liver slices. We had at our disposal only racemic preparations of these amino acids, and so, in order to suppress the action of D-amino acid oxidase, we added sodium benzoate to the samples in concentration of $M \cdot 10^{-2}$ to $M \cdot 10^{-3}$ [16]. As is evident from Table 1, the value of the deaminations of L-ornithine and L-methionine is relatively high. However, we must remark that for methionine nonoxidative deamination is possible under the influence of enzymes of the type of γ-thionase (with formation of CH_3SH, NH_3, and α-ketobutyric acid). Finally, we observed deamination of glycine which proceededintensely in turkey liver and kidney and much less actively in pigeon kidney. In the other objects of study deamination of glycine was practically absent. Analysis of the data of Table 1 draws attention to the particularly high activity of turkey tissues with respect to deamination of a series of L-amino acids and glycine.

We note in the above results that the amount of deamination of the L-amino acids varies greatly depending on the type of tissue and the species of bird. We should also note the great individual variation in intensity of deamination of most of the amino acids in the experiments with any one tissue. Thus, for example, the amount of deamination of L-glutamic acid in pigeon liver varies from experiment to experiment between 4 and 44 μmole per g of tissue. Deamination of aspartic acid in chicken kidney varies from 30 to 136 μmole per g of tissue, and alanine in chicken liver from 4 to 45 μmole per g of tissue. Considering these great individual variations, we must give to the average values shown in the tables only a very relative, orienting significance.

When we compare our data with those of the literature with respect to deamination of a series of L-amino acids in mammalian tissue slices we observe that in bird tissues most of the natural amino acids are deaminated much more actively than in mammalian tissues. In bird liver and kidney slices, in distinction to mammalian, aromatic L-amino acids and L-diamino acids are deaminated.

Systematic experiments on study of the intensity of deamination of unnatural amino acids and racemic forms of amino acids in bird tissue slices were not carried out by us. Individual results in this direction are given in Table 1.

Deamination of L-Amino Acids in Homogenates of Bird Liver and Kidney

We also studied deamination of some natural amino acids in homogenates of liver and kidney of pigeons and chickens and in liver homogenates of turkeys. Our first attempts to carry out deamination of L-amino acids in homogenized liver and kidney tissues were unseccessful.

After a series of trials, we found that the necessary condition for deamination of amino acids in homogenates is the maintainance of a suitable pH in the incubating mixture. For deamination of most amino acids it is necessary to keep the pH above 7.6 and for some amino acids (for example, for the diamino acids) even above 7.8-8.0.

As is known, when homogenates are prepared, the tissue suspension rapidly becomes acid. To avoid this we used a salt medium for preparing the homogenate which consisted of the Ringer-Krebs phosphate solution, 0.1 M, pH 8.5. In this case, during incubation the pH of the sample changed to 7.6, that is, the optimum for deamination of most amino acids. The amount of tissue in the samples varied from 100 to 200 mg in 2 ml total volume of sample. Each sample contained 50 μmole of amino acid. Incubation was carried out for 2 hours in an O_2 atmosphere.

The results of this series of experiments, given in Table 2, show that the amount of deamination of several amino acids in the homogenates does not fall below that in the slices, and for some amino acids (L-leucine,

TABLE 2

Amount of Deamination by Bird Liver and Kidney Homogenates (increase in N-NH_3 in μmole per g of tissue in 2 hours *)

Amino acid	Pigeon		Chicken		Turkey
	liver	kidney	liver	kidney	liver
L-Glutamic acid	17.3	—	9.1	6.5	19.2
L-Aspartic acid	11.0	11.2	8.4	7.8	22.4
L-Alanine	18.6	10.8	18.5	13.4	3.2
L-Valine	2.7	—	4.3	16.8	0
L-Leucine	1.9	—	20.5	12.3	13.0
L-Phenylalanine	5.5	5.6	15.0	17.2	33.6
L-Tyrosine	0	—	9.3	—	0
L-Tryptophan	0	—	14.0	—	29.5
L-Lysine	0	0	18.1	—	33.6
L-Arginine	3.6	14.8	—	—	51.2
Glycine	—	—	—	—	0.3
D-Alanine	26.8	—	—	—	—
DL-Alanine	33.2	—	—	—	—
D-Phenylalanine	—	—	—	74.5	—

* Average of 6-15 experiments.

L-tryptophan, L-lysine in chicken liver) it is even higher. It is interesting that deamination of glycine in turkey liver homogenate, in distinction to the tissue slice, does not occur at all under our experimental conditions. As is known from the literature, in mammalian tissue homogenates there is usually no appreciable deamination of L-amino acids without addition to the incubation mixture of the corresponding cofactors (for example, ATP, cozymase, α-ketoglutarate) [12].

Deamination of Amino Acids in Extracts of Acetone-treated Turkey Liver Tissues

It was also of interest to determine how the deamination of L-amino acids occurs in extracts of acetone-treated liver tissue.

For these experiments acetone-treated preparations of turkey livers were obtained in the cold. The liver tissue was ground three times with cooled acetone (4 volumes each time), then washed with ether and dried

TABLE 3

Amount of Deamination of Amino Acids by the Extract of Acetone-treated Bird Liver Tissue (increase of N-NH_3 in μmole in the sample during 2 hours)

Amino acid	Turkey	Chicken	Pigeon	Amino acid	Turkey	Chicken	Pigeon
L-Lysine	12.7	6.4	0	L-Alanine	0	—	—
L-Arginine	14.2	2.9	0	L-Aspartic acid	0	—	—
L-Histidine*	—	—	—	L-Glutamic acid	2.8	—	—
DL-Ornithine**	8.7	—	—	DL-Methionine**	3.9	—	—
L-Phenylalanine	3.9	—	0	Glycine	0	—	—
L-Tyrosine	0	—	—	D-Phenylalanine	6.2	—	11.2
L-Tryptophan	8.3	—	0				

* In the presence of EDTA.

** In the presence of sodium benzoate.

in a vacuum desiccator. The dry acetone-treated powder could be kept for months in the refrigerator without marked loss of activity. For the experiments, 2 g of dry powder was ground with 14 ml of pyrophosphate buffer, M / 30, pH 8.2. The suspension was centrifuged, the supernatant liquid was separated (extract) and used in the experiment. In the sample the total volume of 2 ml usually contained the extract of 125 ml of dry powder and 20 μmole of amino acid. Incubation lasted 2 hours at 38° in an atmosphere of O_2.

The results obtained in experiments with liver powder extracts are given in Table 3. The extract from acetone-treated turkey liver deaminated best of all the diamino acids: L-lysine, and L-arginine, L-ornithine, and also L-histidine. If we calculate that in the sample there was 20 μmole of substrate, then deamination in the case of lysine and arginine averaged 65-70%. Least, but still at a sufficiently marked rate was the deamination of methionine and phenylalanine.

In the experiments with ornithine and methionine we used the racemates of these acids and added 10^{-2} M sodium benzoate to the samples to suppress the activity of D-amino acid oxidase. To inhibit the action of histidase we added Na_2-ethylenediaminetetraacetate (EDTA) [17] to the samples in which we determined the oxidative deamination of L-histidine.

Our results on the deamination of L-diamino acids, L-histidine, and L-phenylalanine in the extracts are close to the results given for the same amino acids by Boulanger and Osteux [7]. We also succeeded in showing the deamination of L-tryptophan by the extracts of acetone-treated turkey liver. The amount of deamination of L-tryptophan in these experiments averaged 48%. As Table 3 shows, deamination of the other L-amino acids and glycine by extracts of turkey liver did not occur. In Table 3 we give for comparison the results of experiments on the deamination of some amino acids by extracts of chicken and pigeon livers. The activity of the extracts of chicken livers in respect to deamination of L-lysine and L-arginine is lower than in the extracts of turkey liver. The extracts of pigeon livers are not active at all; none of the studied natural amino acids is deaminated under these conditions. Thus, in the experiments with extracts we confirmed the results of Boulanger and Osteux and also for the first time showed the intensive deamination of L-tryptophan in extracts of acetone-treated turkey liver.

SUMMARY

We have carried out a systematic investigation of the deamination of 13 L-amino acids and glycine in slices and homogenates of the livers and kidneys of birds (pigeons, chickens, and turkeys). We have shown that the amount of deamination of L-amino acids differs greatly depending on the organ and species of bird. In bird tissues the intensity of deamination of most L-amino acids is higher than in mammals. We have confirmed the literature data on the deamination of L-diamino acids, L-histidine, and L-phenylalanine in extracts of acetone-treated turkey livers.

We have shown for the first time the intensive deamination of L-tryptophan, characteristic of turkey liver tissues. The difference in relative rate of deamination of the amino acids permits the assumption of presence in birds of different paths of deamination of L-amino acids. Investigation of the mechanism of deamination of the individual amino acids in bird tissues will be continued.

Received December 8, 1957

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** Original Russian pagination. See C.B. Translation.

INACTIVATION OF THE SH-GROUPS OF TISSUE PROTEINS OF RATS IRRADIATED WITH X-RAYS

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In spite of a large amount of work on the role of SH-groups in the development and prevention of radiation sickness, this question is still unsettled. The hypothesis of Barron, according to which selective inactivation of SH enzymes is regarded as the basic mechanism of the biological action of ionizing radiation, has not been confirmed experimentally [1,2]. The mechanism of protective action of SH preparations has been reviewed [3]. The absence of lowering of the activity of sulfhydryl enzymes in the early period of radiation sickness has made unlikely the original idea that the protective action of these compounds is due to blocking the active products of the radiolysis of water.

However, recently data have been obtained which show that inactivation of SH-groups in animal tissues after irradiation does occur and can be observed very soon after irradiation. It is true that there is no basis for thinking that this is connected with a decreased activity of any of the known enzyme systems.

Graevskii and Korchak [4] found a decreased content of SH-groups in the water extract of liver and spleen homogenates of irradiated rats beginning from the third hour after irradiation. Scherer [5] noticed a decreased amount of nonprotein SH-groups in liver tissue an hour after irradiation. Kedrova [6] showed that the amount of SH-groups in the soluble liver proteins of rats decreased even in the first hour after irradiation and continued low for nearly five days after irradiation. In rats which had received a protective injection of cysteine before irradiation, the decrease in amount of SH groups in soluble liver protein was less apparent. Injection into rats of adrenocorticotropic hormone (ACTH) before irradiation increased the percentage of animals which survived the irradiation [7] but did not prevent a decreased content of SH-groups. These results were obtained by the ferricyanide method which determines only the "free" SH-groups of the protein molecule. According to the classification of Barron [8] undenatured proteins contain in addition to the "free" SH-groups, "slowly reacting" SH-groups which are not oxidized by ferricyanide, but can form mercaptides.

To explain the nature of the factors which inactivate the SH-groups of tissue proteins in irradiated animals we were interested in comparing the changes in both types of SH-groups. In this connection we determined in the present work the content of SH-groups in the soluble proteins of rat liver and spleen after irradiating the animals with specific doses of roentgen rays, using two parallel methods: the method of Mirsky and the method of amperometric titration of SH-groups with a solution of silver nitrate. We were also interested in the question of the effect on these changes not only of protective doses of cysteine and ACTH, but also of simultaneous use of both substances. As Kedrova and Krekhova [7] showed, the simultaneous use of ACTH and cysteine, which separately have considerable protective effect, causes more rapid death of irradiated rats than of the controls.

Thus we have the possibility of comparing changes in content of "free" and "slowly reacting" SH-groups in the tissues of rats which had been irradiated without any supplementary treatment, or of using the two protective measures, or of raising the sensitivity of the animals to ionizing radiation. These results we believed would give a basis for deciding the question of the value of inactivating the SH-groups of tissue proteins in the mechanism of radiation sickness.

MATERIALS AND METHODS

The work was carried out on male rats weighing 180-200 g; in all we used about 600 animals.

Irradiation was carried out in a RUM-3 apparatus under the following conditions: 195 kv 15 ma 0.5 mm Cu and 1 mm Al filter, skin focus distance 30 cm, strength of dose 56-60 r/min. The rats were subjected to one total irradiation dose of 650-700 r. For screening the area of the liver we used a lead screen with diameter 2 × 4 cm and thickness 2 mm. In these experiments the rats were irradiated with a dose of 1150 r. Samples for determination of the SH-groups in the tissue proteins were taken after 1 hour, and then daily after irradiation.

The animals were killed by decapitation, the abdominal cavity was quickly opened and a reversed stream of cooled Ringer solution was perfused through the inferior vena cava. When the liver and spleen were sufficiently cleared of blood (as determined by visual inspection of the color of the organ) these organs were removed and homogenized in a glass homogenizer with cooling. Each liver was homogenized separately; four spleens were homogenized in each experiment. The proteins were precipited from the resulting homogenates by acetone in the cold. The resulting acetone-treated preparation was dissolved in a solution of M/60 sodium pyrophosphate (pH 8.3) at 38° and shaken constantly for 45 min. After contrifuging, the protein solution was dialyzed against physiological saline for 18 hours in a refrigerator. In the resulting protein solution the SH groups were determined in parallel by two methods: the method of Mirsky [9] by reaction with ferricyanide, and the method of amperometric titration with 0.001 M silver nitrate solution in an ammonia buffer containing 0.25 M ammonia and 0.01 M NH_4NO_3 [10].

The protein content of the solutions studied was determined by Kjeldahl analysis of the method of Lowry [11].

An aqueous solution of cysteine hydrochloride was injected intraperitoneally 15 min before irradiation, or in the control rats 15 min before taking samples at a dose of 90 mg per 100 g weight of the animals. ACTH was injected intramuscularly in the course of 6 days before irradiation at a dose of 1 unit per animal per day.

The content of SH-groups in the tissue proteins was calculated in micromoles per gram of nitrogen and in % cysteine in respect to the protein. The content of "slowly reacting" SH-groups was calculated by difference from the result obtained by the method of amperometric titration and the Mirsky method.

RESULTS

1. Irradiation Without Supplementary Treatment

As Fig. 1 shows, the method of amperometric titration (AT) gave higher values for the content of SH-groups in the soluble liver and spleen proteins than the Mirsky method. This, as stated above, was because the AT method determines not only the "free" but also the "slowly reacting" SH-groups of the protein molecule. Both methods gave similar results for the change in quantity of SH-groups in tissue proteins in irradiated animals. Inactivation of protein SH-groups which decreased their quantity in liver and spleen proteins could be observed even 1 hour after irradiation of the animal. In the following days the quantity of SH-groups continued to decrease and reached a minimum in 4-5 days. The quantity of SH-groups in liver protein determined by the AT method 1 hour after irradiation decreased by 17% of the original content, and by the Mirsky method, by 18%. If the initial decrease in quantity of SH-groups was expressed in micromoles per gram of protein nitrogen, then the value by the AT method was 102 and by the Mirsky method, 63. This indicates that at the time of irradiation or soon after for each gram of nitrogen of soluble liver protein 63 μmole of "free" and 39 μmole of "slowly reacting" SH-groups were inactivated and became incapable of determination. In latter days the AT method gave a more complex curve of content of SH-groups in liver protein than did the Mirsky method. However, in both cases a change in quantity of SH-groups in liver proteins was found for 4-5 days after irradiation. In this period the amount fell compared to the starting value by 48-57%, or, correspondingly, by 251 (for the AT method) and 203 (for the Mirsky method) expressed in μmole per 1 g of protein nitrogen. This indicates that in 4-5 days after irradiation for each gram of protein in the liver there is inactivation of 203 μmole of "free" and 48 μmole of "slowly reacting" SH-groups.

Thus, immediately after irradiation the quantity of SH-groups in liver proteins decreased because of inactivation in both categories, while in the later period further inactivation of the "slowly reacting" groups almost ceased to occur and decrease in the total amount of SH-groups determined took place because of the "free" groups.

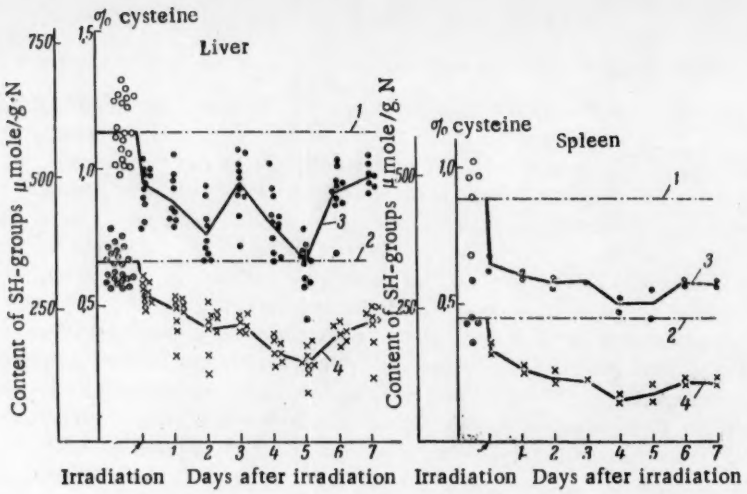

Fig. 1. Content of SH-groups determined in soluble liver and spleen protein of rats in normals and after irradiation of the animals with doses of 600-700 r: 1) average content of SH-groups in soluble proteins of the organs of control rats, determined by the method of amperometric titration (separate experiments indicated by white circles); 2) the same, determined by the Mirsky method (separate experiments indicated by crossed circles); 3) average content of SH-groups in the soluble proteins of the organs of irradiated rats, determined by amperometric titration (separate experiments indicated by black circles); 4) the same, determined by the Mirsky method (separate experiments indicated by crosses)

This was shown still more clearly in the case of the spleen. The amount of SH-groups in soluble spleen protein 1 hour after irradiation compared to the initial value decreased by 125 μ moles per g N when determined by the AT method and by 57 μ moles per g N when the Mirsky method was used. It is apparent that at the time of irradiation or very soon afterward for each gram of soluble spleen protein nitrogen 57 μ moles of "free" and 68 μ moles "slowly reacting" groups were inactivated. In the following days both curves parallel each other from which we can conclude that in the soluble proteins of the spleen, as in those of the liver the "slowly reacting" SH-groups are only inactivated at the time of irradiation or very soon after it. Further decrease in total quantity of SH-groups occurs by inactivation of "free" groups.

II. Irradiation after Injection of Protective Doses of Cysteine

In this series we introduced experiments in which we determined the amount of SH-groups in rat liver protein 30-60 min after a single injection of cysteine (180 mg per rat) without following irradiation. We studied

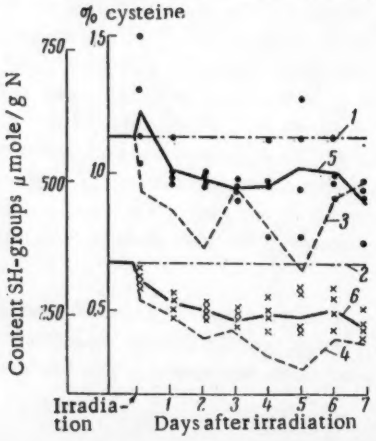

Fig. 2. Content of SH-groups in soluble liver protein of rats irradiated after injection of a protective dose of cysteine: 1, 2, 3, 4) the same as in Fig. 1; 5) amperometric titration method; 6) Mirsky method.

10 animals. In no case did the quantity of SH-groups in the soluble liver proteins exceed the variation in control values after cysteine injection.

Irradiation of the rats 15 min after injection of the cysteine caused a lessenened amount of lowering in quantity of SH-groups in liver proteins as compared to animals irradiated without preliminary injection of cysteine (Fig. 2). Decrease in quantity of SH-groups in liver protein in 1 hour after irradiation in this series of experiments was 9% of the starting value when determined by the Mirsky method and did not differ at all when determined by the AT method. In the following days after irradiation the average maximum decrease in amount of determined SH-groups in soluble proteins of rat liver in this series of experiments was 102-97 μ moles per 1 g M, independent of the method of determination.

Thus we can conclude that the presence of excess cysteine in the animal organism at the time of irradiation prevents the inactivation of the "slowly reacting" SH-groups, and considerably lessens the inactivation of the "free" SH-groups of soluble liver protein.

III. Irradiation of Rats after Injection of ACTH

In this series of experiments we also determined SH-groups in soluble liver proteins in unirradiated rats after injection of ACTH.

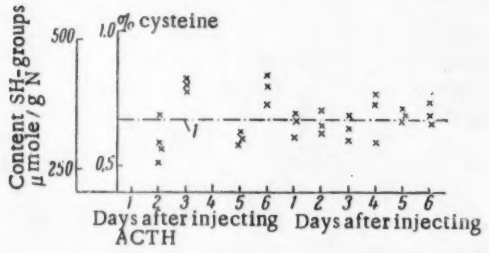

Fig. 3. Content of SH-groups determined in soluble liver proteins in unirradiated rats at the time and after injection of ACTH. Mirsky method: crosses denote separate experiments. 1) the same as in Fig. 1.

As Fig. 3 shows, during injection of the preparations the SH content of the liver proteins of the animals varies within the limits of the controls. After stopping injection of ACTH the SH-group content in the soluble liver proteins did not differ from the control.

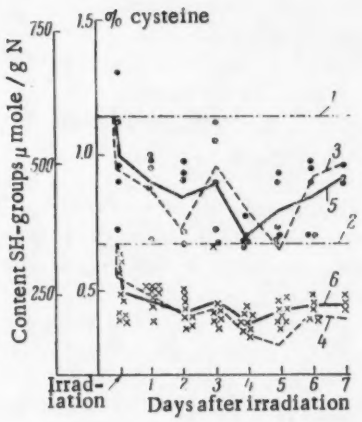

Fig. 4. Content of SH-groups determined in soluble rat liver protein, irradiated after injection of ACTH: 1, 2, 3, 4) the same as in Fig. 1; 5) method of amperometric titration, 6) Mirsky method.

Figure 4 gives the change in quantity of SH-groups in rat liver proteins which were irradiated after injection of ACTH. As Fig. 4 shows, the decrease in amount of SH-groups in this case occurs almost the same as in rats not protected before irradiation. The introduction of ACTH apparently does not affect the rate of inactivation of SH-groups of soluble proteins after irradiation.

IV. Irradiation after Simultaneous Use of ACTH and Cysteine

Figure 5 shows the change in quantity of SH-groups in liver proteins after injection of cysteine into rats which have first received ACTH. As is clear from Fig. 5, one hour after injecting the cysteine both methods show a considerable increase in the determination of SH-groups. Later, the content of SH-groups in tissue proteins gradually returns to normal.

Figure 6 shows the change in content of SH-groups in the liver protein of rats which obtained ACTH and were irradiated 15 min after injection of cysteine. From Fig. 6 it is evident that the quantity of SH-groups determined in this case is already sharply decreased 1 hour after irradiation. When the AT method is

used, the quantity of SH-groups is decreased by 236 μ moles per g N compared to the original content, and by the Mirsky method, by 195 μ moles per g N. If we compare these results with the values obtained by irradiating

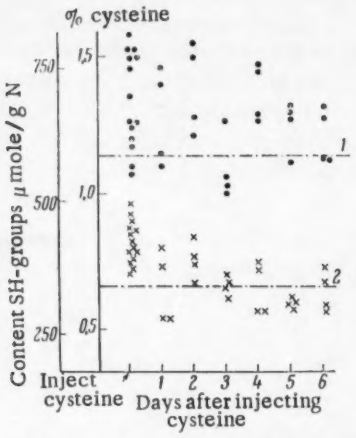

Fig. 5. Content of SH-groups determined in soluble liver proteins of rats which have received ACTH, after cysteine was given to them: 1 and 2 the same as in Fig. 1. The black circles denote separate determinations by the amperometric titration method, the crosses, separate determinations by the Mirsky method.

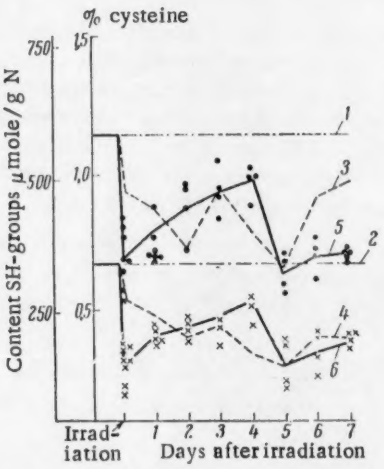

Fig. 6. Content of SH-groups determined in soluble liver proteins of rats, irradiated after injection of ACTH and cysteine: 1, 2, 3, 4) the same as in Fig. 1; 5) by amperometric titration, 6) by the Mirsky method.

animals which had not received either cysteine or ACTH beforehand, we can see that the simultaneous administration of cysteine and ACTH before irradiation sharply increases the initial inactivation of SH-groups. In this case, irradiation produces at once the same decrease in SH-groups which was observed in the first series of experiments only 5 days after irradiation. The sharp decrease in quantity of SH-groups in soluble liver protein in 1 hour after irradiation in rats which received ACTH and cysteine occurs because of increased inactivation of "free" SH-groups, while the decrease in quantity of "slowly reacting" groups is the same in both cases and equals about 40 μ moles per g N of soluble liver protein. In the days following irradiation the content of SH-groups in liver protein of rats which received ACTH and cysteine increases somewhat, but at 5 days falls sharply again.

V. Irradiation with Screening of the Liver Area

We showed above that decreased quantity of SH-groups in liver and spleen tissue proteins could be noted even in the first hour after irradiating the animal. As Fig. 1 shows, this initial inactivation of the SH-groups occurs considerably more intensely than the inactivation which occurs on the following days. In order to determine to what extent the initial inactivation of the SH-groups of tissue proteins is connected with the direct action of ionizing radiation on the organs, we carried out experiments whose results are given in Fig. 7.

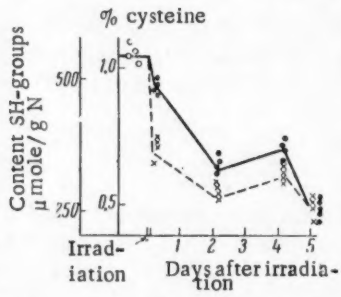

Fig. 7. Content of SH-groups determined in soluble rat liver proteins after irradiating the animals with 1150 r (dotted line) and in animals irradiated with the same dose with the liver area screened (solid line).

As Fig. 7 shows, screening the liver area in rats almost completely prevents the initial inactivation of SH-groups, although in this series of experiments the irradiation dose was increased to 1150 r. In control animals irradiated with this without screening the liver, the quantity of SH-groups determined in the soluble protein of this organ fell more than 30% of the starting value during the first hour after

irradiation. In the days following irradiation, decrease in the SH-groups in the screened rats occurred somewhat more quickly even than in the controls. At the moment of death of the rats (the animals were sacrificed 4-5 dyas after irradiation) the quantity of SH-groups determined in the liver tissue proteins was almost the same in both groups of rats. This indicates that the initial inactivation of the SH-groups of the tissue proteins was connected with the direct action of ionizing radiation on the tissue, while the decreased quantity of SH-groups in the days following irradiation could occur in organs which had not been directly exposed to irradiation.

DISCUSSION

Our results can be summarized as follows. Irradiation causes decrease in quantity of SH-groups determined in liver and spleen tissues of the irradiated animals. Inactivation of the "slowly reacting" SH-groups occurs only at the moment of irradiation or very soon after it and is connected with the direct action of the ionizing radiation of the tissue; it can be prevented by injecting a protective dose of cysteine before irradiation. Inactivation of "free" SH-groups of the tissue protein occurs not only at the time of irradiation or very soon after it, but also for several days following. The injection of cysteine before irradiation decreases but does not prevent the inactivation of "free" SH-groups. Cysteine injected before irradiation into animals which had previously received ACTH not only does not protect the SH-groups of tissue proteins, but, on the contrary, permits their more rapid inactivation.

The differing rates of inactivation of SH-groups in tissue proteins during and after irradiation, and also the fact that inactivation of the "slowly reacting" SH groups is connected with the direct action of the rays on the tissues leads us to believe that agents which inactivate SH-groups at the time of irradiation and in the days which follow are different. Probably inactivation of tissue protein SH-groups at the time of irradiating the animal is connected with the presence in the tissue at this moment of active radicals which are formed under the influence of the irradiation. Since their period of existence is short, these substances cannot diffuse from the irradiated part of the body into unirradiated organs; this also explains the absence of early inactivation of SH-groups in screened tissue. The decreased quantity of SH-groups determined in tissue proteins in the days following irradiation is apparently the result of their inactivation by some sort of less active agent which has a longer life and is able to diffuse or be carried with the blood from one organ to another. Such an agent could be an organic peroxide whose presence in the tissues of irradiated mice has been observed for several days after irradiation [12].

However, if this explains the mechanism of decreased quantity of SH-groups in the tissue proteins of irradiated animals, the question arises, why is the quantity of SH-groups inactivated at the time of irradiation comparatively small, when free radicals are present in the tissues and are very active chemically. When rats are irradiated at doses of 650-700 r, only about 20% of the total SH-groups in the soluble liver protein are inactivated at the time of irradiation. This cannot be explained by a lack of active products of the radiolysis of water, since in rats which have received ACTH and cysteine a considerably greater amount of inactivation of tissue protein SH-groups occurs at the same dosage of x-rays. We must assume that at the moment of irradiation of normal animals who had received no supplementary treatment, most of the so-called "free" SH-groups of the tissue proteins are protected from the action of the inactivating agents. Such an assumption is perfectly reasonable. An increased radioresistance of SH compounds by previous blocking of SH-groups has been shown experimentally [13]. Also, it is difficult to admit that such active and approachable groups as the "free" SH-groups of proteins can remain free long in such a multicomponent system as living protoplasm. We now assume that the "free" and "slowly reacting" SH-group are distributed on the surface of the protein molecule and that they do not form intramolecular bonds [14,15]. This indicates that these SH groups form bonds by which they are protected from the action of inactivating agents. Obviously, these should have the character of intermolecular bonds. A number of experimental facts indicate this. Pasynskii and Cherniak [16] showed that the reactive ability of protein SH groups could be decreased by the participation of these groups in intermolecular bonds. The participation of these groups was shown in the formation of complex protein molecules [17, 18], in lipid–nucleoprotein complexes in the chromosomes of frog eggs [19], and in complexes of glutathione with serum proteins [20].

The idea of the participation of SH-groups in intermolecular bonds and their protection from the action of free radicals formed at the time of irradiation may also explain the contradiction between the results of experiments on inactivation of SH-enzymes when their solutions are irradiated in vitro and the absence of decreased activity of the same enzymes in the irradiated animal. Evidently, in the uninjured tissues of normal animals the SH-groups of the sulfhydryl enzymes are protected from the action of free radicals.

The biological value of intermolecular bonds in tissue proteins may vary. In particular, these bonds should determine the fine molecular structure of protoplasm and cell formation which are characteristic of living tissue. Thus, Mezia [21] considers that because SH-groups form intermolecular bonds in protein molecules, the structure of the mitotic apparatus of the cell is determined. Zagury [19] and Idelman [22] have noted that SH-groups play a part in the formation of lipid – nucleoprotein complexes in the chromosomes of frog eggs. Judging from our data we believe that the intermolecular bonds formed by tissue proteins of liver and spleen are unstable. This is indicated by the fact that inactivation of the protein SH-groups of these organs proceeds gradually, over several days. This agrees well with the data of Eldjarn and his co-workers [23], who on the basis of experiments with labeled cystamine and cysteamine concluded that the protective action of these preparations was determined not by their ability to be acceptors of active radicals, but by their ability to block temporarily the unprotected SH-groups of the protein molecule. It is known that the protective action of both preparations does not last longer than 30-60 min after their injection into the animal. Hence, after this period the bonds of the protein with the injected substances are split, the SH-groups of the protein molecule again become free and are available for the action of inactivating agents.

According to this point of view, we must expect that every action or physiological state of the organism in which the number of unprotected protein SH-groups in the tissues is increased should increase its radiosensitivity. In this respect, the recent work of Idelman [22] is of interest; he showed by histochemical methods that in the chromosomes of cells during mitosis there was an appreciable increase in the quantity of free SH-groups which apparently was connected with the splitting of the lipid – nucleoprotein complexes which he had observed. It is well known that cells during mitotic activity are especially sensitive to irradiation. The increased radiosensitivity [7] and parallel increase in quantity of SH groups of tissue proteins has also been found by us in rats which have received ACTH, in the period of 30-60 min after injecting cysteine.

The mechanism by which the increased SH-groups of tissue proteins are produced when ACTH and cysteine are injected into an animal cannot now be positively explained. However, some data permit us to assume that in this case there occurs displacement of the SH-groups of the tissue proteins from the intermolecular bonds which they have formed, Roberts [24] has shown that injection of ACTH into rats leads to labilization of liver protein. From this we can conclude that injection of ACTH into animals is somehow connected with a change in the properties of the active groups of the protein molecule which share in the formation of intermolecular bonds, and the labilization of these bonds. The splitting of the intermolecular bonds and the freeing of the groups formed from them evidently does not then occur. This is apparent because the quantity of SH-groups of the rat tissue protein, produced only by ACTH, is not increased and their inactivation after irradiation of animal is the same as in the control. However, the properties of the protein SH groups depend to a considerable degree, as is known, on the structure of the molecule in which they occur and can evidently be so changed that the SH-group of cysteine can displace them from those bonds which they have formed. In this connection, the quantity of unprotected SH-groups in rat tissue protein after giving ACTH and cysteine is raised and they are easily inactivated after irradiation of the animal.

On the basis of these data and their discussion we consider it possible to express an opinion concerning the significance of inactivation of SH-groups of tissue proteins in the mechanism of radiation sickness.

The inactivation of tissue protein SH-groups which occurs at the time of irradiation by the action of the radiolysis products of water and also for a longer or shorter time after irradiation under the influence of substances like organic peroxides can lead to destruction of intermolecular bonds in the protein molecules and disorganization of the biological structures in the irradiated organism. This destruction of the fine molecular morphology of the cells of the irradiated organism can play a determining part in the development of the many changes in the metabolism of substances which are found in the later stages of radiation sickness. The idea that the first mechanism of biological action of radiation must be sought in the destruction of a biochemical reaction which determines the submicroscopic organization of the living cell has often been expressed in the literature, particularly by Kuzin [25], Pasynskii [26], Timofeev-Resovskii [27]. We assume that inactivation of the SH-groups in tissue proteins is one of the mechanisms by which the energy of irradiation, accumulated by the active products of radiolysis of water produces a destructive biological effect. This assumption, as it seems to us, agrees well with the findings of Zherebtsov and Bak [28] who studied the early morphological changes in the tissue of irradiated animals and the effect on these changes of a protective injection of SH preparations and also with the idea of Eldjarn [23] concerning the mechanism of protective action of cystamine and cysteamine.

In connection with this idea it is interesting to study the processes of inactivation of tissue protein SH-groups in animals irradiated with different doses of ionizing radiation. We can except that there will be a definite relation between the rate and degree of inactivation of the tissue protein SH-groups of some organs and the death rate of the animals. We also need a study of the action of ionizing radiation on the other active groups of the protein molecule which can share in the formation of intermolecular bonds.

SUMMARY

We have determined the content of SH-groups in the soluble liver and spleen proteins of rats after the action of roentgen rays on the animals. We have found that irradiation causes a decreased quantity of SH-groups determined in liver and spleen proteins of the irradiated animals. The inactivation of the "slowly reacting" SH-groups which occurs only at the moment of irradiation or immediately thereafter and is connected with the direct action of the ionizing radiation on the tissues can be prevented by the injection of protective doses of cysteine before irradiation. Inactivation of the "free" sulfhydryl groups of the tissue proteins occurs not only at the time of irradiation or very soon afterwards, but also for several days following. The injection of cysteine before irradiation lowers but does not prevent the inactivation of the "free" SH-groups. Cysteine injected before irradiation of animals which have first received ACTH, not only does not protect the SH-groups of tissue proteins, but on the contrary, permits their more rapid inactivation. We have expressed the opinion that inactivation of SH-groups of tissue proteins is one of the reasons for the destruction of intermolecular bonds of the protein molecules and the disorganization of biological structures in the irradiated organisms.

Received December 12, 1957

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A HISTOCHEMICAL STUDY OF THE NUCLEOPROTEIN FRACTIONS OF CELL NUCLEI

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At the present time a considerable amount of information has been gathered on the classification of nuclear nucleoproteins, chiefly based on their solubility.

According to the work of Zbarskii and co-workers [1] isolated cell nuclei can be fractionated into nucleoproteins removed by M NaCl, acid proteins, removed by dilute alkali, and residual proteins which are not soluble in the ordinary protein solvents. The nucleoprotein fraction obtained in this way contains desoxyribonucleic acid (DNA), histones, and non-histone, tryptophan-containing proteins. It is identical with the chromosin described by Mirsky and Pollister [2]. The acid protein evidently corresponds to "chromosonin" of Stedman [3]. and the proteins of the "chromosome residue" which contain some ribonucleic acid (RNA) [4]. Finally, Pollister and Leuchtenberger [5],and later other authors [6] described the removal from the nucleus by 0.14 M NaCl of a globulin fraction which contained part of the RNA of the nucleus and some enzymes.

On the other hand, the question of how the cytological formations correspond to one or another of these fractions is not at all clear. It is well known that DNA is present in the chromatin nucleus and RNA in the nucleolus and perhaps in other parts of the nucleus [7]. The distribution of the histones corresponds in general to distribution of DNA in the nucleus [8]. There is no exact and authentic information as to the other protein fractions. Attempts have been made to determine the protein composition of nuclear structures by extraction of sections with NaCl solutions of different concentrations,and then staining. However, the extractions were made either from thick (100 μ) or previously fixed sections [2, 9], and the results showed this.

A number of authors have contended that fractions removed in vitro by this or that solvent do not exist in the nucleus. Thus, Dounce considers that the DNA extracted by M NaCl is an artifact which depends on the action of desoxyribonuclease and trypsin-like enzymes of the mitochondria on the bond of DNA with acid protein. The author observes that when the nucleus is separated under milder conditions the removal of DNA is difficult [6, 10]. The addition of dilute alkali to such a nucleus leads to gel formation [11, 12]. However, the first case can also be explained by the condition of the nuclear membrane, on which, perhaps, depends the extractability of the soluble components. The phenomenon of gel formation can depend on the bond of DNA, not with acid proteins, but with tryptophan-containing proteins of the nucleoprotein fraction.

In the present work we have tried to define more accurately the cytological localization of the nuclear nucleoproteins, using their extraction from sections. For this purpose the sections should be obtained from unfixed tissue so that the nuclear proteins are as close as possible to their native state. In order to have the sections cut through the nuclear membranes to make removal of the soluble components easy, we have also tried to make them very thin.

METHODS

As the chief object of study we used livers and Ehrlich carcinoma of mice, which were growing subcutaneously. Also, in several experiments we used other objects: pancreas, small intestine, kidney, spleen, spinal cord, spinal ganglion, and hepatoma of mice; livers of adult rats and rat embryos; liver and spinal cord of guinea pigs.

We obtained sections of unfixed tissue 3-4 μ thick on a freezing microtome with a cooled knife. The knife was cooled by a curved tube which carried carbon dioxide to the knife. The sections from the knife were transferred to glass and then either fixed directly with absolute alcohol for 2 min, or first extracted with 0.14, 0.4, or 1.0 M NaCl at 0-2° from 2 to 48 hours. Staining the nucleic acids was carried out with methyl green-pyronin at pH 4.7 according to Jordan and Baker [13] with a ribonuclease control; of DNA, according to Feulgen; of protein, according to Mazia with bromphenol blue [14]. In addition we used a combination of staining for DNA and protein: after the Feulgen reaction, where HCl was replaced by trichloroacetic acid, the sections were stained with bromphenol blue.

In addition to the studies of extraction from the sections we carried out a histochemical study of the results of extraction from isolated nuclei of mouse and rat liver, obtained by the method of Chauveau, Moule, and Rouiller [15]. Homogenization was carried out in a glass bacterial homogenizer in 2.2 M sucrose, precipitation took place at 18,000 g for 1 hour with cooling. After separation, the nuclei were extracted with 0.14 M NaCl for 30 min, M NaCl for 18 hours, and 0.05 N NaOH for 30 min; after each extraction a smear was stained with methyl green-pyronin.

RESULTS OF THE INVESTIGATION AND DISCUSSION

Under the influence of any of the above solvents there is extraction of considerable amounts of RNA and protein from the cytoplasm. The 0.4 M NaCl has the strongest action. We can therefore say that if part of the cytoplasmic RNA is adsorbed on the nuclei when they are isolated, it will enter either the globulin fraction if extraction is begun with 0.14 M NaCl, or the nucleoprotein fraction, if we begin with M NaCl. Extraction goes more fully and rapidly from the cytoplasm of the Ehrlich tumor and embryonic cells of the liver, perhaps because of the weak development of the endoplasmatic system and the free state of the ribonucleoprotein granules.

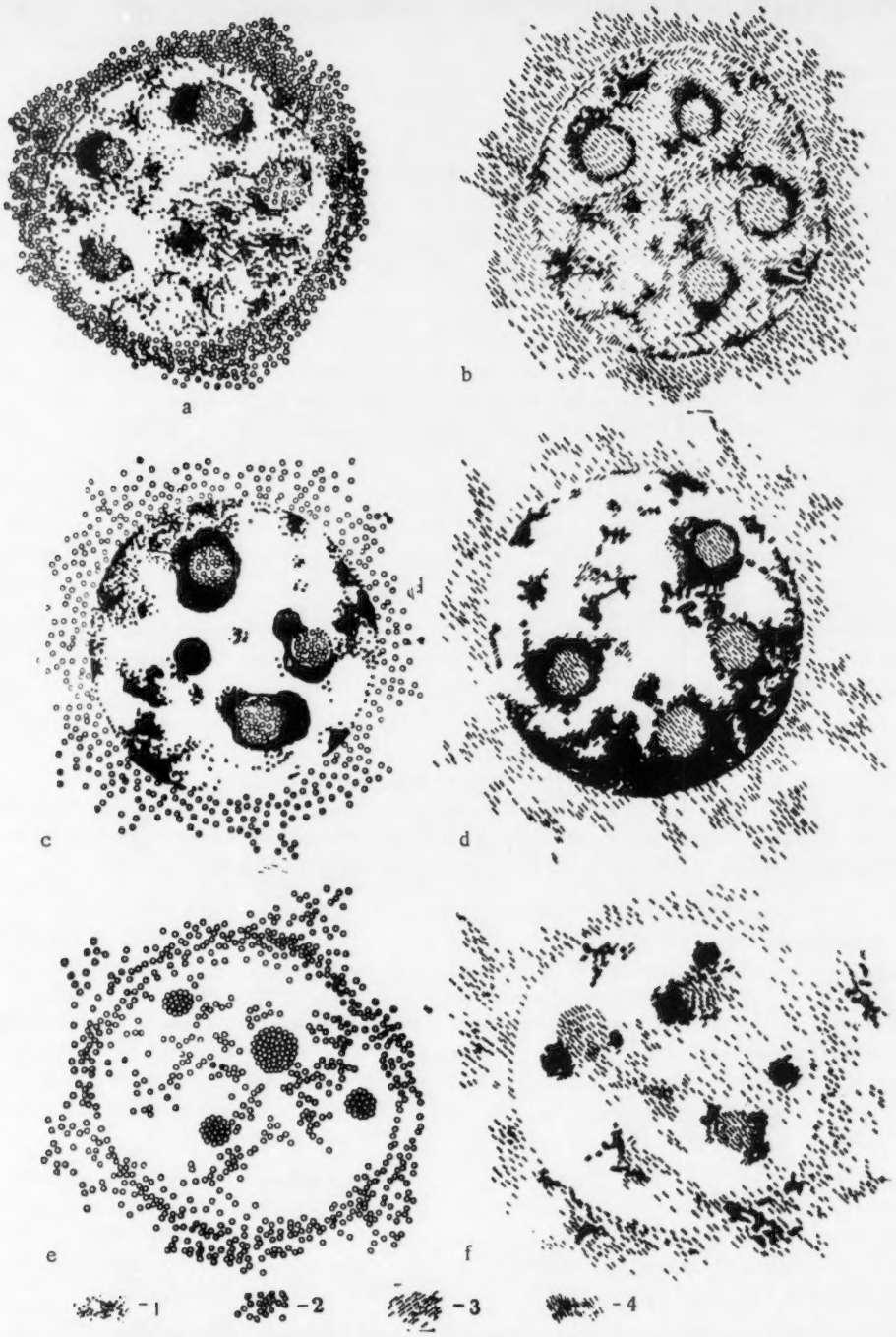
In the unextracted nuclei of liver and Ehrlich tumor were found: nucleoli stained by pyronin (rather pale) and bromphenol blue; chromatin (the perinucleolar chromatin was shown especially clearly), stained by methyl green and the Feulgen method, and the extrachromatin part of the nucleus ("nuclear sap") stained by bromphenol blue.

The 0.14 M NaCl does not remove nucleic acids from the nucleus, to judge by the color of methyl green-pyronin(Fig.c). However, since the latter does not show extrachromatin RNA we can speak only of the chromatin nucleic acids: DNA of the chromatin and RNA of the nucleolus. When we stain with bromphenol blue we observe the loss of a large amount of nuclear protein, and when we stain by the Feulgen method + + bromphenol blue we see that all the extrachromatin protein is removed, since there then appears in the nucleus only the red chromatin (desoxyribonucleoprotein) and the blue nucleolus (Fig. d).

Thus the globulin fraction of the nucleus, removed by 0.14 M NaCl includes all the extrachromatin protein except the protein of the membrane.

M NaCl, usually used for separation of nucleoprotein from acid protein and used directly or after preliminary action of 0.14 M NaCl, deprives the nucleus of the ability to stain with methyl green. Staining of the nucleolus, with pyronin on the other hand, is strengthened, which may be connected with the partial liberation of the phosphate group of RNA. A delicate pyroninophilic network, sensitive to ribonuclease, also appears. In outward appearance it resembles the "chromosome residues" of Mirsky [4] and can be called residual chromatin (Fig. 1, e). Staining with bromphenol blue gives the same result and clearly shows the nucleolus. Thus it follows that ribonucleoproteins of the nucleolus and chromatin are not removed by M NaCl and enter the fraction of acid proteins, since after extraction with alkali they are no longer found. As far as can be judged on the basis of staining with pyronin the amount of RNA in the nucleolus is considerably higher than in the residual chromatin.

When the Feulgen method is used we see that a sufficiently long extraction with M NaCl (1-2 hours) removes all the DNA from the nucleus. The latter sometimes appears as fine fibers precipitated by the fixing agent, stretched out along the glass and showing the considerably viscosity of nucleoprotein solutions. Such a picture is produced only in thin sections where the section passes through the whole nucleus. In thicker sections, where the section does not go through a nucleus, the latter, in spite of long action of M NaCl, retains DNA and only becomes somewhat wrinkled.



Cells of rat livers before and after various extractions: a) No extraction. Stained by methyl green-pyronin; b) the same. Stained by Feulgen process + bromphenol blue; c) extracted with 0.14 M NaCl 15 hours. Stained with methyl green-pyronin; d) the same. Stained by Feulgen process + bromphenol blue; e) extracted with 1 M NaCl 2 hours. Stained with methyl green-pyronin; f) extracted with 1 M NaCl 3 min. Stained by Feulgen process + bromphenol blue. Designations: 1) red stain from binding pyronin; 2) green or blue stain from binding methyl green; 3) blue stain from binding bromphenol blue; 4) red stain from Feulgen reaction.

Thus the extraction of desoxyribonucleoprotein (DNP) by M NaCl is not the result of an autolytic change in the nuclear protein, but depends to a considerable degree on the state of the nuclear membrane (or the cytoplasmic layer which surrounds it). Starting from this we can explain the results of Dounce on the difficulty of extracting DNA by M NaCl from nuclei isolated under mild conditions, which not only consist of nuclear proteins but also the containing membrane.

In the course of the work we considered that the DNA of different parts of the chromatin was not extracted with equal rapidity. Actually, with a short period of extraction by M NaCl even in the first minutes a considerable part of the nuclear DNA was removed, while another part of it remained in the form of granules. In order to show that in this case there was actual removal and not granulation of DNA we placed several drops of M NaCl on the sections and after 2-3 min carefully drew them off and blew them onto a glass slide, added about double the amount of alcohol, and dried them. When we ran the Feulgen reaction on the slide under the microscope we observed fibers which contained DNA. When we stained the above-described granules by the Feulgen process + bromphenol blue, in most cases we could identify them with the perinucleolar chromatin (Fig. f). With longer extraction (15-30 min or more) they even gradually lost DNA. On the basis of the relative stability of perinucleolar chromatin to M NaCl we can assume that it consists of DNP in whose composition occurs the non-histone tryptophan-containing protein.

The 0.4 M NaCl removed extrachromatin protein and left the ribonucleoprotein of the nucleolus, whose staining by pyronin increased; the chromatin DNA was washed out and only the perinucleolar chromatin retained its structure. Staining the latter with bromphenol blue then showed a higher concentration of protein than even in the nucleolus.

The experiments with isolated nuclei gave the same results as those with sections: 0.14 M NaCl did not remove DNA of the nucleus and RNA of the nucleolus; M NaCl removed DNA of the nucleus, leaving RNA, and dilute alkali removed all the nucleic acids.

It is interesting to compare these histochemical data with the results of chemical determinations of the distribution of nucleic acids in the nucleoprotein fractions of isolated cell nuclei carried out by Saidov [16] on mouse liver and hepatoma and the parallel studies which we made on liver (5 experiments), metastases of stomach cancer in human liver (2 experiments), and M-1 rat sarcoma (3 experiments). The nuclei were isolated by the method of Zbarskii and Perevoshchikova [17]. Fractionation of the nuclei and the quantitative analysis of nucleic acids were carried out by the method used in the work of Saidov [16].

It was shown that DNA was found almost entirely in the nucleoprotein fraction (97-98% for human liver; 88-95% for human stomach cancer; and 90-95% for rat M-1 sarcoma). A small part of the DNA was found in the acid protein; this could depend on the presence of undestroyed cells in the preparation, the extraction of which was difficult. Actually, there was a close enough proportionality between the percent of DNA found in the acid protein and the number of undestroyed cells. RNA was distributed between the nucleoprotein fraction and the acid protein (human liver: 42-82% in the nucleoprotein, 18-58% in acid protein; stomach cancer: 40-42% and 58-60% respectively; M-1 sarcoma: 26-35% and 65-74% respectively). The content of RNA in the acid protein was 2-4% in human liver, 4-7% in stomach cancer, and 3-4% in M-1 sarcoma. In the experiments of Saidov on mouse liver and hepatoma similar results were obtained.

During 1957 a number of works appeared [18-20] which showed that nuclear RNA is contained in the globulin fraction and the acid protein fraction. Evidently, in the experiments of Saidov and in ours the RNA passed from the globulin fraction into the nucleoprotein fraction, as did the RNA from the cytoplasmic contamination, since preliminary extraction by 0.14 M NaCl was not carried out in these experiments. The experiments were carried out for the purpose of the above-described histochemical investigation.

Finding RNA in the acid protein connects it still more with the chromosome residues of Mirsky.

The results of the histochemical determination of nucleic acids after extraction fully agree with the previous biochemical data.

On the basis of analysis of the literature data and our own results, we can give the following scheme of cytological characteristics for the nuclear nucleoproteins (table).

The globulin fraction includes all the extrachromatin protein of the nucleus except the membrane protein. Nucleoprotein comprises the chief mass of chromatin, and its extractability by M NaCl is not the result

of autolysis. Probably part of the DNA is bound to histones and part to tryptophan-containing proteins of the nucleoproteins, but not to the acid proteins, since it is appreciably separated from the latter structurally. The tryptophan-containing proteins seem to be concentrated chiefly in the perinucleolar chromatin. The latter is shown by our data on the great stability of perinucleolar DNP to salt extraction; the comparison of the uptake of amino acids in nuclear proteins in vivo and in vitro [21, 22]; with data on autoradiography of amino acids [23] and, finally, the report of the high content of tryptophan in perinuclear chromatin of nerve cells [24].

Cytological Characteristics of Nuclear Nucleoproteins and Proteins

Fraction	Characteristic solvent	Cytological form
Globulin fraction: ribonucleoproteins, enzymes, etc	0.14 M NaCl	Extrachromatin protein
Nucleoproteins	M NaCl	Chromatin (chiefly general chromatin*)
(a. DNA-histone)*		(Chiefly perinucleolar chromatin*)
(b. DNA-tryptophan-containing proteins)*		
Acid protein (ribonucleoprotein + lipoprotein)*	0.05 N NaOH	Nucleoli + "residual chromatin"
Residual Protein		Nuclear membrane

* The statements in parentheses are hypothetical.

The acid protein, according to the data given, contains proteins and ribonucleoproteins of the nucleolus and residual chromatin. Comparison of the chemical data [25, 26] with electron-microscopic studies of the nucleolus [27, 28] shows its heterogeneous composition (ribonucleoproteins + lipoproteins). Finally, the residual protein is the protein of the nuclear membrane [29].

SUMMARY

We have studied the intranuclear localization of the nucleoprotein fractions extracted from isolated nuclei by different solvents. For this purpose we have carried out extractions with salt solutions of different concentrations on unfixed sections 3-4 μ thick, obtained with a freezing microtome using a cooled knife, followed by staining of the sections for nucleic acids and proteins.

The globulin fraction, removed by 0.14 M NaCl, includes all the extrachromatin proteins ("nuclear sap").

M NaCl quickly removes desoxyribonucleoproteins (DNP) from the chromatin of the nucleus through which a section has been cut, but if the membrane is preserved, extraction is difficult.

Thus the solubility of the nuclear DNP in M NaCl is not an artifact produced by autolysis.

The acid protein removed by dilute alkali includes RNA, nucleolar protein, and "residual chromatin".

On the basis of the great stability of perinucleolar chromatin to salt extraction and from the literature data, we have assumed that chromatin DNP contains chiefly non-histone tryptophan-containing proteins.

Histochemical study of isolated nuclei submitted to salt extraction gave analogous results.

We express thanks to Prof. I. B. Zbarskii for suggesting this subject and for valuable advice.

Received December 30, 1957

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THE ASSIMILATION OF THIAMINE-S^{35} SULFUR BY THE PROTEINS OF THE ANIMAL ORGANISM

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The irreversible splitting of vitamin B_1 in the organism to non-vitamin products is one of the chief factors which determines the requirements of this vitamin, and a basic reason for endogenous hypovitaminosis B_1.

The study of the splitting of thiamine to non-vitamin products has become possible only since the availability of labeled atoms. In a number of studies [1-5] carried out with thiamine-S^{35} it has been shown that part of the thiamine sulfur is oxidized in the human and animal organisms to sulfate, which is excreted in the urine.

The formation of sulfate when vitamin B_1 splits indicates that sulfur is separated from the thiamine molecule or its metabolite during interstitial metabolism. However, sulfate is the end-product in the oxidation of sulfur, and it seemed very probable to us that the irreversible splitting of vitamin B_1 in the animal organism would form compounds which contained reduced sulfur. Such a path for the splitting of vitamin B_1 is quite possible if we calculate that thiamine in the animal organism is easily converted to the thiol form with formation of SH groups and disulfides as has been shown by a number of foreign [6-10] and native investigators [11].

To demonstrate this assumption, we decided, in our first studies [12, 13] to follow the fate of the reduced sulfur which formed in the splitting of thiamine by determining its distribution in such sulfur-containing compounds of the animal organism as amino acids and proteins. When rats are injected parenterally with thiamine-S^{35}, the proteins isolated from organs contain radioactive sulfur which makes up a considerable percentage of the total sulfur of the organ (from 1.81-7.82%). The S^{35} content in the protein depends to a considerable extent on the deposition of the injected thiamine-S^{35} in the organs of white rats; this makes it difficult to estimate the intensity of assimilation of thiamine sulfur by the proteins.

The present work is devoted to the intensity of assimilation of thiamine-S^{35} sulfur by the tissue proteins as related to the dose of vitamin injected. Also in this work we describe experiments to show that labeled sulfur found in the proteins of rats which have received thiamine-S^{35} enters the sulfur-containing amino acids and is not the sulfur of thiamine-S^{35} bound in some form to the proteins.

I. Study of the Intensity of Assimilation of Sulfur by Tissue Proteins of White Rats after Injection of Various Doses of Thiamine-S^{35}

The study was carried out with 25 young male white rats weighing 130-160 g on a ration of 12 g of "casein milk" (which contained, in %, protein 17-22, fat, 14-16, lactose, 13-17, sucrose, 35-39, ash, 3-4, and moisture, 8), 0.2 g dried yeast, 0.2 ml fish oil, and 40 ml water. Thiamine-S^{35} was injected once subcutaneously (activity 50,000 counts/min per g animal weight). A definite amount of non-radioactive thiamine was always added in the injection (bearer) so that the total dose of vitamin B_1 injected into the animal was 50 or 500 μg. As our previous experiments showed [13], with these radioactivities of injected thiamine-S^{35} we could obtain preparations of $BaS^{35}O_4$ from the tissue proteins sufficiently active to count on an end-window counter. At 24 hours after injecting thiamine-S^{35} we decapitated the animal and at once the liver, kidney, small intestine, brain and muscle were taken for investigation. A sample of the organs was carefully ground in a mortar

in the cold and the protein was extracted from it by the method described by Konnikova, Kritsman, and Iukhnovskaia [14]. For separation from the S^{35} of thiaminepyrophosphate, the protein was hydrolyzed with 0.25 N HCl for 20 min at 100° by the method of Widenbaner [15]. After hydrolysis the main mass of protein was separated by centrifuging and the dissolved protein from the hydrochloric acid extract was precipitated with trichloroacetic acid. The protein precipitates were combined and again purified by the method of Konnikova et al. It should be noted that in our previous work [13] preliminary extraction of the protein was not carried out; the ground tissues were hydrolyzed and then the protein was extracted. After the isolated protein had been burned by the method of Benedict [16] the sulfur was isolated in the form of $BaS^{35}O_4$ and its radioactivity measured on the type B end-window counter with account being taken of the autoabsorbed β-rays [17].

TABLE 1

Intensity of Assimilation of Sulfur by Tissue Proteins when Rats Are Injected with Different Doses of Thiamine-S^{35}

Dose of thiamine-S^{35} in μg	Number of animals	Organ studied	Radioactivity of proteins extracted from 1 g of tissue in counts / min	S^{35} uptake in the protein in %
50	10	Liver	281 (232—331)	0.58
		Kidney	166 (98—200)	0.33
		Small intestine	102 (61—173)	0.20
		Brain	165 (122—217)	0.33
		Skeletal muscle	54 (26—116)	0.11
500	10	Liver	493 (402—598)	0,98
		Kidney	715 (600—841)	1.43
		Small intestine	225 (166—262)	0.45
		Brain	255 (209—316)	0.51
		Skeletal muscle	72 (40—120)	0.14
5 mg unlabeled thiamine and in 24 hours, 500 μg thiamine-S^{35}	5	Liver	786 (625—882)	1.57
		Kidney	1153 (1016—1270)	2.30
		Brain	346 (288—441)	0.69
		Small intestine	366 (335—412)	0.67

Note: We give average data from 10 or 5 experiments, and also the minimum and maximum values (in parentheses).

We estimated the intensity of assimilation of thiamine-S^{35} sulfur by tissue proteins from the ratio of radioactivity of the proteins extracted from 1 g of tissue to the radioactivity of injected thiamine-S^{35} per g of live weight of the rats (in %).

When we analyze the results given in Table 1 we can see that the radioactivity of the proteins extracted from different organ and tissue proteins of rats is not the same and depends on the dose of injected thiamine-S^{35}, whose radioactivity in all experiments was the same. With a single injection of white rats with 50 μg thiamine-S^{35} (which fell within the limits of an optimum dose) from 0.11 to 0.58% of its sulfur was assimilated by the proteins of different organs.

According to radioactivity, the proteins of the studied tissues can be arranged in the following order: liver > kidney and brain > small intestine > muscle. The intensity of sulfur assimilation when thiamine was injected in doses of 500 and 5500 μg was higher and relatively more of it was destroyed in a organism. In this case the maximum percent sulfur uptake in proteins of the liver was 2.3% but in the whole organism thiamine sulfur was assimilated less, since the intensity of its uptake in the proteins of other organs and tissues was several times lower.

According to radioactivity of the proteins of the rat organs which we studied, when 500 μg thiamine was injected, the following order can be given: kidney > liver > brain > small intestine > skeletal muscle. The high radioactivity of kidney proteins in this case can be explained by the presence in the kidney of a large amount

of thiamine-S^{35} and its metabolites, excreted in the urine, which produces more energetic assimilation of radiosulfur by kidney proteins.

The differing ability of the proteins of the organs and tissues which we studied to assimilate thiamine sulfur can be explained not only by an unequal distribution of vitamin B_1 in the organs and tissues.

We can also consider the differences in rate of renewal of the amino acid composition of the proteins. According to their rate of renewal the tissue proteins are distributed in the same sequence (kidney> liver> brain> > skeletal muscle [14, 18-20]) as in their ability to assimilate thiamine-S^{35} sulfur.

2. Study of the Nature of the Labeled Compounds Found in the Proteins Isolated from the Organs of Rats Which have Received Thiamine-S^{35}

The proteins extracted from the organs of rats which have received thiamine-S^{35}, proteins which contain radiosulfur, have been used by us to show that this sulfur enters the sulfur-containing amino acids and is not thiamine-S^{35} absorbed on the protein surface or precipitated on them by trichloroacetic acid, or S^{35}-thiaminepyrophosphate incompletely split by apoenzymes during the hydrolysis of the proteins by hydrochloric acid, or any other metabolite of thiamine which contains radiosulfur in some form bound to protein.

This question could best be solved by using paper chromatography for the analysis of the the sulfur-containing amino acids in the protein hydrolyzate with a count of the radioactivity along the strip. However, this was impossible practically because of the weak radioactivity of the analyzable part of the protein hydrolyzate which was deposited on the paper. Further increase in dose of thiamine injected to reach the desired radioactivity of the tissue proteins might produce radiation sickness in the white rats, since the radioactivity of the injected thiamine-S^{35} would greatly surpass the indicator dose assumed for S^{35} [21].

A better method under our conditions for determining the sulfur-containing amino acids (especially cystine) in the proteins which we obtained is the extraction of cystine from them in the form of copper mercaptide. For this purpose the proteins extracted from liver and kidney of white rats which had received 500 μg of thiamine-S^{35} were submitted to 20-hour hydrolysis with 10 volumes of a mixture of 18% hydrochloric acid and 20% formic acid (1 : 1) by the method of Bolling and Block [22] in which cystine is not destroyed. Cystine was precipitated quantitatively from the protein hydrolyzate with copper chloride by the method of Wilken-Jorden [23]. The copper mercaptide isolated from the protein hydrolyzate, and also the initial protein, were burned according to Benedict and a precipitate of barium sulfate was obtained in which radioactivity of the S^{35} was calculated.

TABLE 2

Radioactivity of Cystine-S^{35} Isolated from the Liver and Kidneys of Rats Which had Received 500 μg of Thiamine-S^{35} (number of rats, 10)

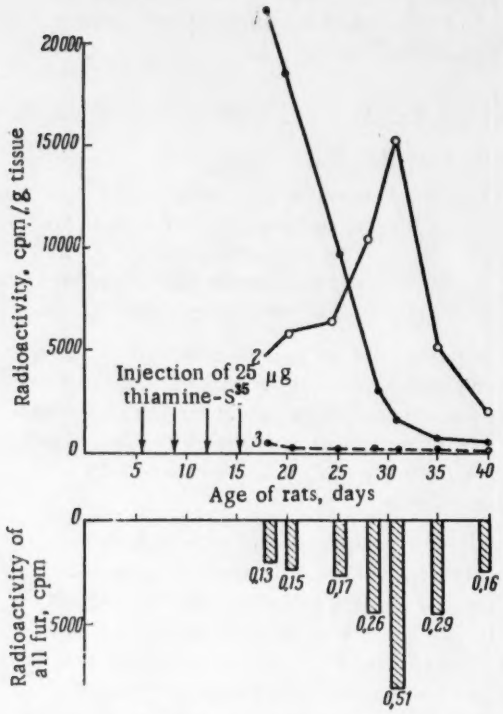
Organ studied	Radioactivity in counts / min per g tissue		S^{35}-cystine in % of total S^{35}-proteins
	S^{35}-proteins	S^{35}-cystine	
Liver	502 (411-588)	168 (107-206)	33.4%
Kidney	724(633-850)	300(131-415)	41.4%

A comparison of the radioactivity of the protein and the cystine-S^{35} isolated from it per g of tissue is given in Table 2.

The resulting data (Table 2) show that in proteins isolated from organs of white rats 24 hours after injecting them with 500 μg of thiamine-S^{35} there is cystine-S^{35}, and in proteins extracted from the liver its content averages 33.4% in respect to total S^{35} protein, and in kidney protein, 41.4%. These results are satisfactory

evidence of the possibility of using thiamine sulfur for endogenous formation of sulfur-containing amino acids.

Interesting data which characterized the intensity of assimilation of thiamine sulfur in the biosynthesis of sulfur-containing amino acids and their deposition in the organism were obtained by us in a study of the radioactivity of the fur of growing young rats which had been repeatedly injected with thiamine-S^{35}.



Assimilation of S^{35} by the fur of growing young rats under conditions of prolonged injection of thiamine-S^{35}. In all we injected 100 μg of thiamine-S^{35} with an activity of 1,568,000 counts/min (taken as 100%). The figures under the columns are the activity of the radiosulfur assimilated by all the fur of the animals, in % of the total activity of the injected thiamine-S^{35}. 1) Liver, 2) fur, 3) blood.

For the experiment we used newborn rats of three litters (in all, 25 rats). Rats of one litter, beginning on the sixth day (before any fur had appeared), were injected intramuscularly with 25 μg of thiamine-S^{35} every three days. In all, four such injections of thiamine-S^{35} were made with a total dose of 100 μg with a radioactivity of 1,500,000 counts/min in each rat. Three days after the last injection of thiamine-S^{35} when the rats were already covered with fur, we took from the studied litter one rat each 3-5 days to determine the content of radiosulfur in the fur, liver, and blood. The rats were decapitated and the blood collected. After removing the tails and feet we took the skin and fur which were washed for 24 hours in running water, then stretched on a glass cylinder and in this form washed successively for 30 min with alcohol, a mixture of alcohol and ether, and ether. After drying the hides at room temperature, we shaved off the fur, weighed it, and burned part of it by the Benedict method. In the same way we burned the blood and liver. The radioactivity of the resulting precipitates of barium sulfate was calculated as in the previous experiments.

The results from one of three experiments are given in the figure, in which the upper part shows the change in radioactivity in counts/min per g of fur, liver, and blood, and the lower part of columns indicates the radioactivity (counts/min) of radiosulfur found in all the fur of the animal. The figures show the comparison of percent of radiosulfur assimilated by all the animal fur with the total dose of thiamine-S^{35} injected (taken as 100%).

The data show that the radioactive sulfur injected into white rats as thiamine-S^{35} is assimilated by the fur proteins. It is interesting that assimilation of the vitamin sulfur by the fur continues to increase for many days after injection of thiamine-S^{35} has stopped. Thus, for example, the radioactivity of the total fur in the rats 3 days after injection of thiamine-S^{35} has stopped is 0.13% of the injection activity of thiamine-S^{35} (lower part of the figure), and after 16 days the radioactivity of the total fur has increased almost four times to about 0.51%. Afterwards the radioactivity of the fur decreases due to loss and replacement of hair. When we compare the radioactivity per g of fur, liver, and blood in rats killed at different periods after injections have been stopped (upper part of figure), we see that the radioactivity of the liver tissue is very high on the first day and quickly decreases to 10-15 days after injections have stopped. This can be explained by removal of thiamine-S^{35} and its metabolites from the liver, and also by a decreased amount of S^{35}-containing amino acids because of renewal of liver proteins.

The curve of radioactivity of fur has a different character: it rises especially strongly from 10 to 16 days after injections of thiamine-S^{35} have stopped, reflecting the intensity of deposition of the vitamin sulfur in the organism of the growing rat.

The observed radioactive sulfur in the fur of rats which have received thiamine-S^{35} is one more evidence of the possibility of assimilation of the reduced sulfur of vitamin B_1 by amino acids and proteins, since fur contains sulfur almost exclusively in the form of sulfur-containing amino acids, and particularly, cystine [24].

Summarizing all the studies presented in our work, we can consider it established that the sulfur of vitamin B_1, when it splits in the rat organism, is partly assimilated by sulfur-containing amino acids and proteins. This fact indicates the possibility of forming compounds which contain reduced sulfur when thiamine is split in the animal organism. Some assumption of the mechanism of thiamine splitting in the organism with assimilation of the vitamin sulfur by tissue proteins was given by us in previous work [5].

According to contemporary data [6-11], thiamine in animal tissues is reversibly converted to the thiol form with splitting of the thiazole ring and formation of free aldehyde and SH groups. Such a thiol form, which we call "thiamine hydrosulfide" can react with amino acids and thus lose sulfur [5].

Starting from this, we may think that oxidation of thiamine sulfur in the organism proceeds with participation of amino acids and proteins which assimilate the reduced sulfur of thiamine hydrosulfide to form sulfur-containing amino acids and these are further oxidized to sulfate. This assumption is confirmed by our data [5] which indicate the strengthening of the oxidation of thiamine-S^{35} sulfur to sulfate when along with it there is injected a reducing agent capable of forming thiamine hydrosulfide in the tissues. However, the possibility is not excluded of another pathway of assimilating the vitamin sulfur by amino acids and proteins. In particular there is the possibility of the splitting of thiamine in animal tissues by thiaminase [25] into its pyrimidine and thiazole constituents, which can then react by trans-sulfuration with thiamine analogs. An explanation of this path of splitting for thiamine-S^{35} will be the subject of our further investigation.

SUMMARY

When the sulfur of parenterally injected thiamine-S^{35} splits in the organism of white rats it is partly assimilated by tissue proteins and enters the composition of sulfur-containing amino acids. This has been shown by extraction from purified liver and kidney proteins of cystine-S^{35} and also by determination of the content of radiosulfur in the animal fur. The assimilation of vitamin sulfur by tissue proteins can be considerably strengthened by increasing the injected dose of thiamine, which causes destruction of a considerably greater part of it in the organism.

Considering the intensity of assimilation of the vitamin sulfur by tissue proteins, the character of its distribution in the proteins isolated from different organs, and also the intensity of deposition of the vitamin sulfur in the fur, we can consider it established that when thiamine-S^{35} splits in the organism the sulfur is liberated in the reduced form.

We can assume that thiamine-S^{35} is oxidized in the animal organism with participation of amino acids and proteins which assimilate the reduced sulfur of vitamin B_1 or its metabolites with formation of sulfur-containing amino acids which are further oxidized to sulfate.

Received December 31, 1957

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THE CHEMICAL NATURE OF EREMURAN, A NEW POLYSACCHARIDE FROM THE ROOT OF EREMURUS REGELII

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The search for new emulsifying agents for pharmaceutical technology led Ponomareva [1] to the extraction from the root of Eremurus regelii (family Liliaceae) of an "adhesive substance" which resembled gum arabic in external properties. Preliminary experiments carried out by B. N. Stepanenko and O. N. Ponomareva showed that the main part of the "adhesive substance" of Eremurus was a polysaccharide which gave a red color with iodine, like glycogen. This new polysaccharide was called eremuran. Since only a very small number of plant polysaccharides are known which give a red color with iodine, the study of eremuran was of considerable interest. The chemical characteristics of eremuran are also very important in connection with the possibility of using it in medicine and pharmacy (for example, as an emulsifier).

The first of the plant polysaccharides which gave a red color with iodine (aside from the glycogen of lower plants – fungi, yeasts, and bacteria) was described in the last century by the botanists Negeli [2] and A. A. Meyer [2] who observed such polysaccharides in some representatives of the higher plants, orchids and lilies. These authors considered such polysaccharides to be starch, which, however, gave "anomalous" colors with iodine. These observations were later forgotten and it was generally accepted that a red coloration with iodine was specific for polysaccharides of animal origin (glycogen) and a blue coloration was specific for polysaccharides of higher plants (starch). Only in 1939 did Morris and Morris [3] publish work on the polysaccharides of the seeds of one type of corn ("Golden Bantam") which gave a red color with iodine and was therefore called "plant glycogen". Somewhat later Hassid and McCready [4] showed that besides this "plant glycogen" the kernels of this type of corn contained ordinary starch; study by the method of methylation of the "plant glycogen" showed that it resembled the glycogen of animal tissues. The same conclusion was reached by K. Meyer and Fuld [5]. Sumner and Somers [6] isolated "plant glycogen" from two other kinds of corn.

Orchid and lily polysaccharides which give a red color with iodine, according to the observations of Negeli and A. Meyer [2] have not been studied up to now.

Isolation and Properties of Eremuran

The plant Eremurus regelii Vved., which forms great thickets in Central Asia, is widely known there under the name "shiriash" (Uzbek), "sharjash" (Kazak), and "sharysh" (Tadzhik). Eremurus regelii can also grow under the conditions of the central area, especially in the region of Moscow (Chief Botanical Garden, Academy of Sciences, USSR) [7]. The investigation described below was carried out on material from Central Asia.

The Eremurus roots were collected in July in southern Kazakhstan on the Aksu-Dzhabagla Reservation. The fresh roots were cut into cylindrical pieces and dried in the southern sun. As a necessary measure the roots were ground to a fine powder. The powder was extracted with water at room temperature, 1 part powder to 10 parts water.

Successful and rapid separation of the extract from the plant tissue was quite difficult because of its high viscosity and adhesiveness; dilution had other undersirable consequences (increase in cost of alcohol, filtration of large volumes, loss of polysaccharide). We usually filtered the greater part of the extract through linen and obtained a very clear filtrate, and then separated the extract which remained with the tissue by centrifuging.

The eremuran was precipitated from the resulting extract by ethyl alcohol. Separation of large, white, slimy flakes began even when a small amount of ethanol had been added. Full precipitation was reached by adding 3-4 volumes, but double the volume (with respect to the volume of extract), which we usually used, precipitated most of the polysaccharide. After it had stood, the precipitate was separated by centrifuging and the gelatinous precipitate was further sucked dry on a Buchner funnel with a rapid filter.

Purification from contaminating proteins was carried out by one or two reprecipitations with double the volume of alcohol from a solution in 5% trichloroacetic acid. The well-separated precipitate was repeatedly washed with alcohol of increasing concentrations (from 60 to 96%). Final drying was carried out in a desiccator with P_2O_5. Dehydration by alcohol of increasing concentrations was of great value, since if this stage of drying was incomplete, the eremuran formed dark, horny granules which were very difficult to grind and which lost their solubility in water.

The eremuran made up about 30% of the weight of air dried, ground root of this Central Asiatic plant. (According to the data of Ponomareva [8] the content of "adhesive substance" in Eremurus roots grown in the Moscow region was lower).

Well-washed and dried eremuran was a white, light ("dusty") amorphous powder. It dissolved in water in the ratio 1:10 with formation of a sticky-strongly opalescent solution. Solution was hastened by preliminary grinding of the swollen particles with a small amount of water to which more was later added. The viscosity of the eremuran solution surpassed many times the viscosity of a gum arabic solution of the same concentration.

The specific activity of eremuran was $[\alpha]_D^{26} = -32.5°$ (c = 0.98%, l = 1, in 0.1 N aqueous solution of NaOH, with lessened opalescence).

The reaction of the eremuran solution was nearly neutral; the pH of a 1% solution was 6.5.

Explanation of the Chemical Nature and Establishment of the Structural Components of Eremuran

When it was heated with Fehling solution, eremuran showed scarcely noticeable reducing properties. The reducing power of eremuran determined by the Hagedorn-Jensen method was equivalent to about 3% of that of glucose. However, after 3-hour hydrolysis with 2.5% HCl the reducing power became 90-94% of that of glucose (increasing the duration of hydrolysis to 5.7 and 8 hours scarcely increased the reducing power further). The final value for the reducing power of the hydrolyzate indicates that eremuran is a polysaccharide (which perhaps contains a very small amount of admixture), and such a sharp increase in reducing power permits us to consider it a higher polyose or its mixture.

Identification of the structural components of eremuran was carried out by chromatography, conversion of the monosaccharides into their derivatives, and fermentation.

Chromatographic Identification of the Components

The hydrolyzates obtained after 3, 5, and 8-hour hydrolysis with 2.5 or 5% HCl or H_2SO_4 were freed from Cl or SO_4 ions by ion exchange resin MMG-1 or (in hydrolysis with H_2SO_4) by neutralization with $BaCO_3$. Chromatographic analysis was carried out on Leningrad paper and on Whatman No. 2 paper in the presence of a number of sugars as indicators for from 18-20 hours to 5-7 days. As solvents we used phenol – H_2O, collidine–H_2O, n-butanol–acetic acid–H_2O (4:1:5), and benzene – pyridine – n-butanol–H_2O (1:3:5:3). As developers we used aniline hydrophthalate, napthoresorcin, urea, p-aminobenzoic acid, benzidine, $AgNO_3$-NH_3.

Using different combinations of these solvents and developers, in which the clearest results were given by collidine–H_2O and n-butanol–acetic acid–H_2O and aniline hydrophthalate, we were able to show that the structural components of eremuran were glucose and mannose which were present in the greatest amounts, to judge by the intensity of the color of the spots (Fig. 1).

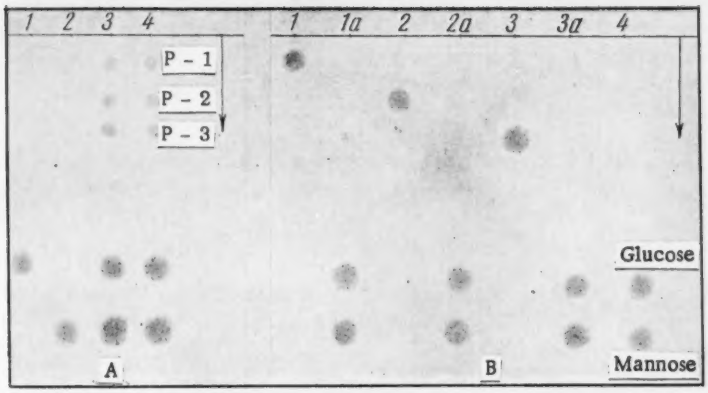

Fig. 1. Chromatogram of eremuran hydrolyzate. Solvent: n-butanol-acetic acid-water; developer: aniline phthalate. A – hydrolysis with 2.5% HCl for 3 hours, 1) glucose, 2) mannose, 3 and 4) hydrolyzate fractions I and II of eremuran; P–1, P–2, P–3 – spots of products of incomplete hydrolysis of eremuran; B – chromatogram of hydrolyzates P-1, P-2, and P-3 isolated from several chromatograms of the type of the preceding one (A); I) P-1, 1a) its hydrolyzate; 2) P-2, 2a) its hydrolyzate; 3) P-3, 3a) its hydrolyzate; 4) mixture of glucose and mannose.

Aside from the intense spots of glucose and mannose, in some cases the paper showed 2-3 other very weak spots (Fig. 1, A). From their position on the chromatogram they could belong to uronic acids or to oligosaccharides, products of incomplete hydrolysis of eremuran. For a solution of this question we carried out the hydrolysis of eremuran under stronger conditions (5-10% H_2SO_4, longer period of hydrolysis). The chromatogram of the hydrolyzate showed sharp weakening in the intensity of these weak intermediate spots.

For a final solution of the question of the absence in eremuran of components other than glucose and mannose we carried out a chromatographic separation of the hydrolyzates in parallel on several sheets of paper, developed the control strips from each chromatogram and cut out the places on the other chromatograms which corresponded to the positions of the weak spots of the developed control chromatogram strips. The cut out parts of the chromatograms were extracted two or three times with water, the concentrated extract was submitted to a further hydrolysis (2.5% H_2SO_4) and the resulting hydrolyzate was again chromatographed.

We observed clearly on the chromatograms from these hydrolyzates glucose and mannose (Fig. 1, B) and either complete or almost complete disappearance of the intermediate spots. These experiments indicate that the intermediate weak spots in some of the chromatograms are not due to uronic acids, but to oligosaccharides, which are intermediate products of hydrolysis and which contain glucose and mannose as components.

Thus, glucose and mannose are the sole structural components of eremuran.

Identification of the Components of Eremuran by Their Derivatives

At the beginning of our work with eremuran we tried to isolate and identify osazones of the sugars from the hydrolyzate. When phenylhydrazine acted in the usual way on the decolorized and neutralized hydrolyzate, we easily obtained a precipitate. After the first crystallization we obtained a substance melting at 182-183°. Five recrystallizations raised the melting point to 190-191°, which did not agree with the melting point of a known osazone; that is, we did not obtain a pure osazone. This is often found when there is a mixture of different sugars with a large number of components. In our case this could be explained also by the presence of mannose which gives an insoluble hydrazone; this evidently contaminated the osazone, which is the same for glucose and mannose.

We had much greater success in obtaining the pure, difficulty soluble phenylhydrazone of mannose in an alcohol medium by the method of Butler and Cretcher [9]

The eremuran hydrolyzate (hydrolysis of 0.1 g eremuran in 1.5 ml 2.5% H_2SO_4) was neutralized with $BaCO_3$ and filtered from the precipitate of $BaCO_3$ and $BaSO_4$, then mixed with a solution of phenylhydrazine base (0.1 ml) in alcohol (0.35 ml). After 15-20 min at room temperature there was formed a snow white, crystalline precipitate. After recrystallization from dilute alcohol the mannose phenylhydrazone melted at 179-181°.

The mannose phenylhydrazone preparation obtained from the hydrolyzate was identified through a preparation of the phenylhydrazone obtained from pure mannose; a mixed melting point gave no depression.

Identification of the Eremuran Components by Fermentation

The polysaccharide (70 mg) was hydrolyzed with 2.5% H_2SO_4, the hydrolyzate was neutralized with $BaCO_3$, and the filtrate was concentrated. Fermentation was carried out with the yeast Schizosaccharomyces Pombe-Linder which selectively ferments glucose and mannose. Fermentation (20 hours, phosphate buffer, pH 5.5) was carried out with:

1 – eremuran hydrolyzate
2 – eremuran hydrolyzate + galactose
3 – a mixture of pure glucose, mannose, and galactose.

At the same time we ran a control of eremuran hydrolyzate and killed yeast. After incubation the filtered incubation mixture was chromatographed. The solvent was n-butanol-benzene-pyridine-water (5:1:3:3); the developer was aniline phthalate.

On the control chromatogram we saw two clear spots for glucose and mannose; in experiment 1 (hydrolyzate) these two spots had fully disappeared; in experiment 2 hydrolyzate + galactose only the galactose spot was observed; in experiment 3 (glucose + mannose + galactose) we also saw only the one spot of galactose.

Thus, the fermentation experiments fully confirmed the earlier data that glucose and mannose are the only components of eremuran.

Fractionation of Eremuran and Study of the Fractions

The discovery in eremuran of two structural components, glucose and mannose, led to the conclusion that eremuran is either a heteropolysaccharide, a glucomannan, or a mixture of homopolysaccharides composed of residues of different monoses. To decide this question we ran a series of attemps to separate eremuran into fractions which might, perhaps, differ in composition.

Fractionation by the Schoch Method

Eremuran which gave a red color with iodine, especially in its main part, is evidently a polysaccharide with a branched chain structure [10]. However, the possibility is not excluded of the presence in it of a small amount of admixture of polysaccharides of linear or slightly branched structure. For this reason we tried to fractionate eremuran by the method of Schoch [11], assuming that in the presence of linear or weakly branched fractions, the complexes of the latter with butanol would precipitate. However, neither by following the exact method of Schoch nor by several variations of it did we obtain a precipitate, which indicates the absence in eremuran of a mixed linear fraction.

Electrophoretic Fractionation

Since recently such difficultly fractionable branched polysaccharides as glycogen have been successfully separated into fractions by paper electrophoresis [12] we tried in this way to separate eremuran. The experiments were carried out in a veronal buffer, pH 8.6, and also in a borate buffer with later oxidation of the polysaccharide (after electrophoresis) by 1% KIO_4 and development by fuchsin and an acid solution of bisulfite.

A series of experiments gave negative results: when the electrophoregram was developed only one colored zone appeared.

Fractionation by Precipitation with Ethanol

If eremuran is a mixture of homopolysaccharides composed of glucose and mannose, connected, perhaps, in different ways, we might expect a somewhat different solubility of these polysaccharides in alcohol and

alcohol mixtures. From this point of view it was interesting to try to obtain fractions of eremuran precipitated at different concentrations of alcohol.

We separated fractions by precipitating with one volume of 96% alcohol and then after separating this fraction (I) adding to the filtrate another volume of alcohol and precipitating fraction II. We thus obtained two fractions precipitated by one and two volumes of alcohol. The quantitative ratio of the two fractions was usually 3:1 or 4:1.

Study of the Eremuran Fractions

In the two fractions separated by the above method we determined: moisture content, ash, phoshorus method of Fiske and Subbarow), nitrogen, reducing power (Hagedorn and Jensen method) before and after hydrolysis, approximate quantitative content of mannose and glucose, and we also studied the iodine reaction.

The approximate quantitative determination of the structural components of the eremuran fractions, glucose and mannose, was carried out by quantitative chromatography.

After running several parallel experiments on paper chromatographic separation of the hydrolyzed eremuran fractions, control strips from each sheet were developed (aniline phthalate) and from the places on the chromatogram corresponding to the positions of the glucose and mannose spots we cut out the pieces of paper and extracted them repeatedly; the extracts served for the quantitative determination of the sugars. As controls we used extracts obtained by removing other parts of the chromatogram outside the spots.

TABLE 1

Characteristics of the Eremuran Fractions

Fraction	No. of volumes of alcohol for precipitation	Moisture	Ash	Phosphorus	Nitrogen	Reducing power calculated as mg glucose		Opalescence of solution	Ratio of reducing power of glucose and mannose	Max. extinction in the iodine reaction
		in %				before hydrolysis	after hydrolysis			
I	1	5.56	0.1—0.2	Trace	0.26	3.1—3.4	93—94	Strong	1:1.9	0.31
II	2	5.01	0.1—0.2	Trace	0.11	6—7.5	91—92	Weak	1:1.9	0.15

The reducing power of the extracts of glucose and mannose and also of the control extracts was determined according to Hagedorn and Jensen and expressed as mg glucose. Further, the reducing power of both sugars (allowing for the values obtained in the control experiments) was expressed in percent of the reducing power of the hydrolyzate used for the chromatogram. The results of these determinations are given in Table 2.

Table 2 shows that in all cases the sum of the reducing power of glucose and mannose is about 90% (on the average, 94%), that is, although it does not reach the reducing power of the hydrolyzate, it approaches it. This fact indicates that the loss in hydrolysis due to formation of the above-mentioned small amount of difficultly hydrolyzed oligosaccharides and also the loss in extracting the chromatogram is very small.

Especially important is the fact that in all the numerous analyses of reducing power, that of the mannose formed in hydrolysis is about twice as much mannose as glucose. For determination of a more accurate quantitative ratio it would be necessary to use the corresponding coefficients for calculating the reducing power of mannose found by the Hagedorn method (worked out for glucose) transposed to mg of mannose. However, we were not able to find such data for the Hagedorn-Jensen method in the literature [13].

By the use of many methods (Folin-Wu, Teffer-Hartman, McLean, Sumner) the reducing power of mannose is shown to be less than that of glucose [14]. Thus, by the Sumner method the reducing power of mannose is 0.74 that of glucose. If the same ratio holds for the Hagedorn method, then the ratio which we find for the reducing powers of glucose and mannose in the hydrolyzate, equal to 1:1.9 should be replaced by a weight ratio 1:2.5.

TABLE 2

Reducing Power of Glucose and Mannose in the Hydrolyzates of Eremuran Fractions

Reducing power in % of reducing power of hydrolyzate			Ratio of reducing power of glucose and mannose	Reducing power in % of reducing power of hydrolyzate			Ratio of reducing power of glucose and mannose
glucose	mannose	glucose + mannose		glucose	mannose	glucose + mannose	
Fraction I				Fraction II			
31.51	65.88	97.39	1:2.1	32.73	61.82	94.19	1:1.9
30.60	60.67	91.27	1:2.0	32.60	61.82	94.42	1:1.9
34.73	57.89	92.62	1:1.7	33.54	61.82	95.36	1:1.8
32.88	56.97	89.85	1:1.7	28.70	61.82	90.52	1:2.1
32.88	62.99	95.87	1:1.9	30.43	63.54	93.97	1:1.2
32.42	59.74	92.16	1:1.8	33.76	63.56	97.32	1:1.9
34.73	62.09	96.82	1:1.8	29.11	57.95	87.06	1:1.9
29.64	59.75	99.36	1:2.1	32.60	57.95	90.58	1:1.8
Average		94.42	1:1.9	Average		92.92	1:1.9

The exact determination of the mannose content of the hydrolyzate by more specific methods is our next problem.

However, as a first approximation we can consider that the mannose content in eremuran is two or more times as great as the glucose content.

As Table 2 shows, both fractions of eremuran have the same ratio of glucose and mannose.

Iodine reaction of the eremuran fractions. Eremuran gives a red color with iodine which disappears with heating and appears again with cooling. Photometric study of the iodine reaction of the eremuran fractions was carried out under conditions resembling those used in the study of glycogen [15]. However, since eremuran gives a less intense color with iodine then does glycogen, we used more concentrated eremuran solutions – 10 mg/ml or higher. The absorption curves of fractions I and II of iodoeremuran and also of glycogen are given in Fig. 2.

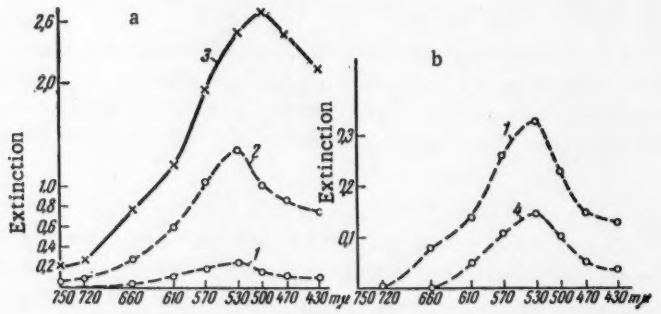

Fig. 2. Absorption curves for the compounds of eremuran and glycogen with iodine: 1) eremuran fraction I (10 mg/ml + 0.1 ml 0.1 N I_2 + + H_2O to 5 ml); 2) the same (25 mg/ml + 0.1 ml 0.1 N I_2 + H_2O to 5 ml); 3) glycogen (10 mg/ml + 0.1 ml 0.1 N I_2 + H_2O to 5 ml); 4) eremuran fraction II (10 mg/ml + 0.1 ml 0.1 N I_2 + H_2O to 5 ml). 1 = 5 mm.

When we collect all the data on the composition and properties of fractions I and II of eremuran (Table 1) we see that, even with many similar analytical results, and, which is very important, with the same content of structural components, eremuran fraction I differs from II in lower reducing power before hydrolysis (3.1-3.4

instead of 6-7.5), stronger opalescence, and much more intense iodine reaction. All these differences do not supply a sufficient basis for assuming different structures for the fractions. In recent years results have been obtained that both linear and branched polysaccharides, amylopectin and synthetic glycogen, with the same type of structure (the same chain units and length of external branches) can give different intensities of color with iodine depending on the degree of polymerization [16, 17]. The differences in the fractions which we observed could be fully explained by different degrees of polymerizations: fraction I, more easily precipitated by alcohol, evidently is a mixture of higher molecular weight polymer homologs and therefore has less reducing power, greater opalescence, and a more intense iodine reaction.

Thus, the three methods which we used to fractionate eremuran did not result in its separation into different types of polysaccharides.

In recent times it has been believed that a color with iodine is given only by homopolysaccharides built from glucose. However, a few years ago Gilles and Smith [18] isolated from wheat flour an as yet insufficiently studied polysaccharide built, according to chromatographic data, from glucose, arabinose, and xylose, which gives a blue color with iodine. It is possible that eremuran is a similar exception, a heteropolysaccharide which gives a color with iodine.

SUMMARY

We have extracted a polysaccharide from the roots of the plant Eremurus regelii Vved. and called it eremuran. The amount of eremuran reaches 30% of the dry weight of the root of this Central Asiatic plant.

The polysaccharide gives a red color with iodine (absorption maximum at 530 mμ), similar to the color of glycogens. The eremuran solution has a high viscosity, $[\alpha]_D^{26} = -32.5°$.

When the acid hydrolyzate is studied chromatographically and by a fermentation method, it shows the presence of glucose and mannose in it. Mannose was also identified by its crystalline phenylhydrazone.

By means of quantitative paper chromatography the ratio of glucose and mannose was found to be ~1:2. Attempts to fractionate eremuran with butanol, and also by paper electrophoresis did not give any results. Two fractions were obtained by precipitation with different concentrations of alcohol. A study of the properties of these fractions and their content of structural components led to the conclusion that these fractions evidently differed only in degree of polymerization. These data indicate that eremuran is a glucomannan.

Received December 31, 1957

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THE SEPARATION OF HIGHER FATTY ACIDS OF PLANT TISSUES BY THE METHOD OF REVERSED PHASE PARTITION CHROMATOGRAPHY

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The adsorption-chromatographic method was developed by M. S. Tswett in 1903 for the separation, on a column, of lipophilic natural substances, pigments. As a result of the further development of this method, processes have been worked out for partition chromatography on filter paper which have the widest applicability for separation of water-soluble substances. The use of paper chromatography in its original form for the separation of natural complex mixtures of lipids was impossible because of the hydrophilic nature of the inert carrier used and the non-mobile phase. Chromatographic separation of lipids was accomplished only after the introduction by Ramsay and Patterson [1] in 1948 of the "reversed phase" principle. According to this principle, the substance under study was dissolved in a non-mobile hydrophobic phase, and separation was carried out by its continuous distribution between the non-mobile phase and a mobile hydrophilic phase. In 1950 Boldingh [2] used for the separation of the methyl esters of higher fatty acids a paper, treated with vulcanized latex, and a mixture of different volumes of acetone and methanol as the solvent; but the spots, after immersion of the chromatogram in a solution of lipophilic dye, were poorly developed against the intensely colored base. The higher fatty acid could not be separated by this method. Early work on the paper chromatographic separation of lipids is given in the review of Holman [3].

The work of Kaufmann [4] occupies a leading position in the development of methods for separation of higher fatty acids on paper. The fatty acids were deposited on the paper in saturated undecane (b.p. 190-220°) and separated by a 70-90% aqueous solution of acetic acid. After both phases were removed from the paper by heating to 120°, the spots were developed by forming their colored copper salts. The method has been varied in later work by other authors [5-9]. Because of the hydrophilic properties of the paper used, the reproducibility of the Kaufmann method in our laboratory has not been good; it has been impossible to obtain even and smooth impregnation of the paper in successive experiments, and the paper swells and tears when it is washed for a long time in running water.

Making the paper hydrophobic has been accomplished in a number of studies by soaking it in a mixture of triglycerides from plant oils [10, 11], silicons [12-14], naphthalene [15], a solution of aluminum potassium alum [16], paraffin [16, 17], kerosine [18], paraffin oil [19, 20], etc. The reactive carboxyl groups have been chiefly used in detecting the acids (formation of metallic salts which give colored sulfides or ferricyanides, change in color of acid-alkali indicators), or else oxidation of double bonds (formation of MnO_2 in oxidation by permanganate, detection of aldehydes formed by ozonization by the Schiff reaction). The chief objection to these methods is the difficulty of developing the spots without first removing the non-mobile phase from the paper.

Since the separation of the fatty acids themselves met with difficulty, attempts were made to chromatograph derivatives of the fatty acids: products of union of mercury with double bonds [21], hydroxamic acids [22, 23], acetoacetic ester derivatives [24], brominated and hydroxylated fatty acids [4], p-(4-nitrophenylazo)-phenacetyl esters [25], derivatives of N-acyl-p-dimethylaminophenyl urea [26], and 2,4-dinitrobenzenesulfonyl chloride [27]. The necessity of obtaining the derivatives before separation required a large amount of starting fatty acids which excluded these procedures from use in micromethods.

As already mentioned, the general characteristic of all these methods is the use of a polar liquid as the mobile phase – one of the three components of all partition chromatogram systems. In the case of a reversed phase system, both the other necessary components – the non-mobile phase and the inert support – should be hydrophobic. In all the studies on distribution of fatty acids on paper, only one of the components was hydrophobic: either the inert support (paper soaked in latex or paraffin or the non-mobile phase (kerosine or Decaline). The other component either was entirely absent or remained hydrophilic.

The basis of the present work is the idea that to gurantee effective separation of a complex mixture of fatty acids, the reversed phase chromatographic system should include all the necessary components, both hydrophilic and hydrophobic.

The hydrophobic inert support in our system is filter paper soaked once in an 0.5% solution of rubber in benzene; as the hydrophobic non-mobile phase we use a fraction of aliphatic hydrocarbons from kerosine (b. p. 220-260°), and as a solvent, 90-99% acetic acid saturated with the same mixture of hydrocarbons. The mixture to be studied, containing about 20-50 μg of each component, was deposited on the surface of the paper which was placed in an ordinary chamber for descending chromatography. The separation process usually lasted 15-18 hours.

After separation, the fatty acids were concentrated in the hydrophobic phase in the dissolved state. However, the reaction of the carboxyl groups which served to identify the spots could occur only in polar solvents. To liberate the carboxyl groups, the high-boiling hydrocarbons were removed from the paper completely by long treatment of the chromatogram with a stream of hot air. The unsaturated acids were fully preserved in this treatment by the moderate temperature of heating. To develop the spots the separate acids on the chromatogram were treated with a solution of $AgNO_3$. The spots appeared when the silver salt was converted to the dark sulfide in a solution of Na_2S.

EXPERIMENTAL PARTS

Saponification of the oil and obtaining a preparation of fatty acids. In a 50 ml beaker made of heat-resistant glass 0.8 g KOH was dissolved with heating in 5 ml of dry glycerol; at the same time 2.1 ml of oil was heated in a flask. When no more water vapor was evolved from the glycerol, the oil was added to it and the mixture was stirred with a glass rod with heating for 1-3 min, until a clear solution formed. After cooling somewhat, 3 ml of 25% H_2SO_4 solution was added dropwise. The layer of fatty acids was drawn off and dissolved in acetone or petroleum ether to obtain a 4% solution.

Oxidation of the unsaturated fatty acids of the oil. In 2 ml of a mixture of acetic acid with acetone (1:3), containing 0.4% $(CH_3COO)_2Hg$ (a catalyst for the oxidation of double bonds) was dissolved 20 ml of fatty acids, and 50 mg of finely powered $KMnO_4$ was added. After full solution of $KMnO_4$ the solution was kept at room temperature for 1-2 hours. Excess $KMnO_4$ was decolorized with several drops of dilute aqueous H_2O_2 and 1 ml of glacial acetic acid was added to the solution. The resulting solution was deposited on the paper. This process oxidized the unsaturated fatty acids completely and the saturated fatty acids remained unchanged.

Obtaining the separate fatty acids used as "indicators". Pelargonic acid was obtained by oxidizing oleic acid with a mixture of KIO_4 and $KMnO_4$ by the method of Rudloff [28] followed by extraction of the liquid products of the oxidation.

Capric acid was extracted from the fatty acids of coconut oil by separation of a large amount of the acids on paper followed by elution from the paper with acetone. In analogous fashion we obtained a pure preparation of lauric acid.

Myristic, palmitic, stearic, and petroselinic acids were chromatically pure commercial acids with melting points close to the theoretical.

Oleic acid (a commercial preparation for determining water hardness) was strongly contaminated by other acids; it was purified by fractional precipitation of its complex with urea until a chromatographically pure preparation was obtained, free from palmitic acid.

As preparations of erucic and eicosenic acids, and also ricinoleic acid we used respectively the fatty acids from mustard oil and castor oil without fractionation.

To obtain a preparation of linolenic acid we used the method of fractional precipitation of fatty acids as their urea complexes [29], since obtaining polyunsaturated acids by debromination of their bromides led to partial isomerization of their double bonds [30]. The method described below allowed us to obtain the natural cis-form of the acid. Since this acid is easily oxidized in air, its methyl ester was fractionated. The ester was obtained by methanolysis of 110 ml of linseed oil (iodine number 179 according to Hanus) in a solution of 300 mg of metallic sodium in 200 ml of absolute methanol for 60 min on a water bath. The clear solution was acidified with 2.5 ml N HCl in 700 ml of hot water; the ester layer was filtered through a paper filter and dried with a rapid stream of heated N_2. The resulting methyl esters were dissolved with heating in a solution of 300 mg of pure urea in 1500 ml of absolute methanol. When the solution was slowly cooled to room temperature a precipitate separated and was discarded; the solution was kept at −5° overnight. The precipitate was separated and the methanol was distilled from the solution until a crystalline precipitate formed. The complex of methyl linolenate with urea was decomposed with hot, dilute HCl and 25 ml of methyl linolenate was obtained with n_D^{20} 1.4720. The preparation was purified by distillation at 172° at 1 mm Hg and saponified as described above. For further fractionation, 80 ml of linolenic acid was dissolved with heat in a solution of 320 g of urea in 400 ml of methanol; the solution was slowly cooled to −2°, the precipitated complex of linolenic acid with urea was filtered on a Buchner funnel and washed with 100 ml of benzene. The weight of the precipitate was 240 g. About 1 ml of linolenic acid was contained in 4 g of the urea complex. The iodine number of the preparation by the Hanus method was 256.7 which corresponded to 90% linolenic acid (100% linolenic acid obtained by reduction of the hexabromide [31] has an iodine number of 273). Linolenic acid can be kept in the form of the urea complex without notable change for a long time and can be isolated from the complex by heating with 10 times the amount of dilute HCl.

Linoleic acid, obtained by the same method from poppy-seed oil, had an iodine number of 172.

C_{16} – C_{24}-fatty acids from peanut oil were separated from unsaturated acids in the form of urea complexes and were dissolved with heat in petroleum ether (b.p. 40-60°) to obtain 1% solutions.

The fatty acids (except those from peanut oil) were deposited on the paper as 2% solutions in acetone.

Purification of the filter paper. Sheets of filter paper from the Volodar Leningrad factory (made in 1953) or the "Goznak" Leningrad factory with dimensions of 4.5 × 55 cm were washed in a descending chromatography chamber with 50% acetic acid until the eluate was colorless, and then dried. Heavy metal salts were thus removed from the paper.

Preparation of the rubber solution. Rubber freshly precipitated from the roots of kok-saghyz alkali process [32] was cut up with a knife and 1.5 g was placed in 300 ml of dry benzene. Solution was considerably hastened by periodic shaking of the mixture and was usually complete in a day. Non-rubber impurities in the form of a flaky precipitate were separated by filtration through a glass filter No. 1. We could also use for this work hevea rubber which had not undergone oxidative destruction.

Preparation of hydrocarbons. Commercial kerosine was distilled in a fractionating column and the fraction boiling from 220 to 260° was collected. The distillate was treated for several days in a separatory funnel on a mechanical shaker with successive portions of concentrated H_2SO_4 until the acid layer after shaking with kerosine was only pale yellow. The aliphatic fraction of the kerosine, freed from aromatics and naphthenes, was washed with water to remove the acid, dried with anhydrous Na_2SO_4 and filtered through a paper filter.

The resulting hydrocarbon mixture had n_D^{20} 0.4451 and was a colorless liquid with a faint, pleasant odor. For the treatment of the paper we prepared a 5-10% solution of the hydrocarbons in acetone.

Preparation of the mobile hydrophilic phase. Glacial acetic acid (b.p. 118°) was dissolved in water to obtain 90 to 99% solutions. Some of the hydrocarbon mixture was added to the solutions and they were carefully heated to 50° with stirring, then left at room temperature until stratification of the phases occurred. The lower phase was used as the solvent.

Course of the chromatographic separation. The washed sheets of paper were saturated with water vapor in a desiccator for 1-2 hours and then passed through the rubber solution; after the excess solution had drained off the strips were hung for 1-3 min for drying and then passed through a solution of hydrocarbons in acetone. After volatilization of the acetone, the strips were placed between two pieces of glass and at a distance of 7 cm from the upper edge were placed 3 drops of fatty acid solution at 1.5-cm intervals. The strips were placed

in an ordinary paper-lined chamber for descending chromatography, in the bottom of which was poured 90% acetic acid and the separation was carried out for 15-18 hours at room temperature without permitting draining of the solvent from the paper. Then the paper was removed and dried to complete removal of acetic acid.

The higher fatty acids with 20-24 atoms of carbon were isoluble in 90% and cold 99% acetic acid. Therefore, to separate these acids, we used the following procedure. The fatty acid solution was deposited on the strip at a distance of 2 cm from its edge; the strip was placed by its upper end in a cylindrical bath for the solvent, such that the spots of fatty acids were inside the upper end of the bath, and the edge of the strip was pressed to the bottom of the bath with a glass rod. Then the 99% acetic acid saturated with hydrocarbons was placed in the bath and heated to 30-40°. The chamber was tightly covered and kept until the front of the solvent reached the edge of the paper strip. The rest of the analysis was the same as usual.

Developing the chromatogram. The dried paper strips were placed in a special blower which supplied a stream of air heated to 30-80°. Heating was regulated by a rheostat. After 2-3 hours the paper was removed, rolled in a spiral and placed in a dark vessel (250 ml) with a 1% $AgNO_3$ solution of 10% alcohol. After 15 min the liquid was poured off and the paper immersed in a 1% $AgNO_3$ solution in 50% alcohol to develop the saturated acids. After another 15 min the silver solution was poured off and the paper repeatedly washed, first with distilled water, then with tap water, and then again with distilled water to remove the excess silver. The washed paper was placed in a 1.5% solution of Na_2S and in the places where the silver salts of the fatty acids were concentrated there appeared dark brown spots of silver sulfide which did not change further. The excess Na_2S was removed with water, the chromatogram was taken out of the vessel and dried. Identification of the individual spots was carried out with the help of the corresponding "indicators."

Values of the R_f Coefficient for Individual Fatty Acids

Common name of fatty acid	Geneva system name of the fatty acid	R_f
Erucic	n-Δ^{13}_{11}-docosenoic (cis)	0.06
Eicosenic	n-Δ-eicosenoic (cis)	0.10
Stearic	n-octadecanoic	0.10
Oleic	n-Δ^{9}-octadecenoic (cis)	0.18
Petroselenic	n-Δ^{6}-octadecenoic (cis)	0.18
Palmitic	n-hexadecanoic	0.18
Linoleic	n-$\Delta^{9,12}$-octadecadienoic (cis)	0.28
Myristic	n-tetradecanoic	0.34
Linolenic	n-$\Delta^{9,12,15}$-octadecatrienoic (cis)	0.39
Lauric	n-dodecenoic	0.52
Capric	n-decanoic	0.69
Pelargonic	n-nonanoic	0.81
Ricinoleic	n-Δ^{9} hydroxyoctadecenoic (cis)	0.92
	High Fatty Acid from Peanut Oil*	
Lignoceric	n-tetracosanoic	0.42
Behenic	n-docosanoic	0.54
Arachidic	n-eicosanoic	0.70
Stearic	n-octadecanoic	0.85
Palmitic	n-hexadecanoic	1.00

*For the higher fatty acids from peanut oil we give the value of R_n (ratio of the distance traveled by a given acid to the distance traveled by palmitic acid).

Figure 1 gives the chromatogram obtained with different mixtures of 13 fatty acids which are found in the composition of widely distributed cultivated plants. The R_f values of the different acids are given in the table.

Figure 2 gives the chromatogram for the five saturated fatty acids from peanut oil (their R_n is given in the table). In view of the poor solubility of these acids we used heated 99% acetic acid for their separation.

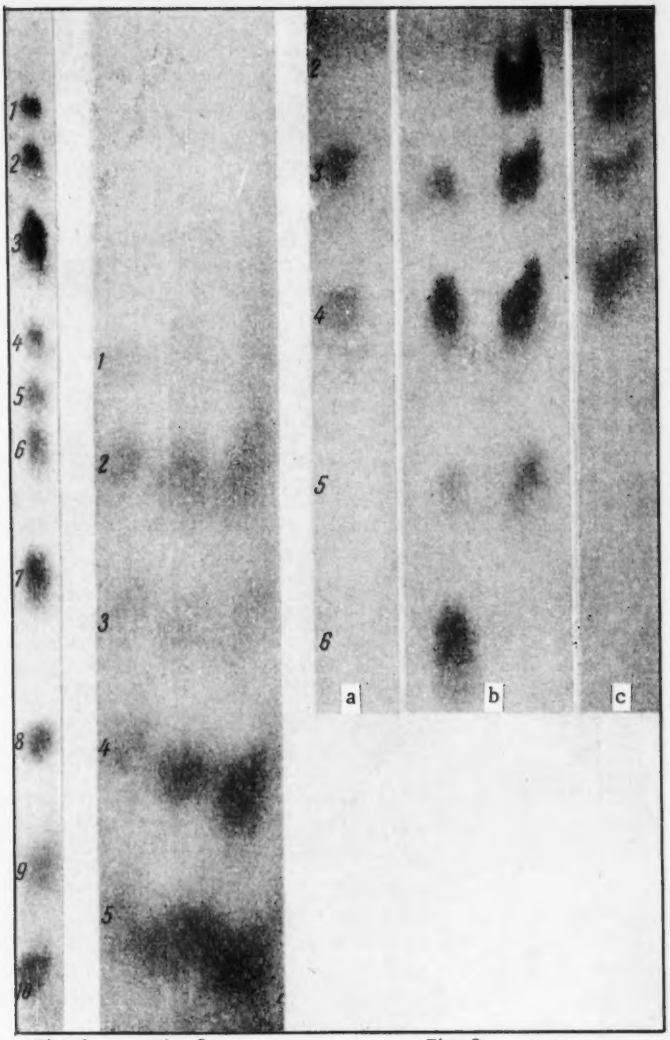

Fig. 1 Fig. 2 Fig. 3

Fig. 1. Separation of a mixture of higher fatty acids on hydrophobic paper. Solvent – 90% acetic acid; developer $AgNO_3$ and Na_2S. Acids: 1) erucic; 2) eicosenic and stearic; 3) oleic, petroselenic, and palmitic; 4) linoleic; 5) myristic; 6) linolenic; 7) lauric; 8) capric; 9) pelargonic; 10) ricinoleic.

Fig. 2. Distribution of the higher saturated fatty acids of peanut oil on hydrophobic paper. Solvent – 90% acetic acid; developer $AgNO_3$ and Na_2S. Acids: 1) lignoceric; 2) behenic; 3) arachidic; 4) stearic; 5) palmitic. Above the spot of lignoceric acid appear three weak spots of as yet unidentified fatty acids, which contain more than 24 carbon atoms.

Fig. 3. Separation of the high fatty acids of linseed and mustard oils. A – Acids of linseed oil: 3) stearic; 4) oleic and palmitic; 5) linoleic; 6) linolenic; saturated linseed acids: 3) stearic; 4) palmitic. B – Acids of mustard oil: 1) behenic; 2) erucic and arachidic; 3) eicosenic and stearic; 4) oleic and palmitic; 5) linoleic; 6) linolenic; b – saturated acids of mustard oil: 1) behenic; 2) arachidic; 3) stearic; 4) palmitic.

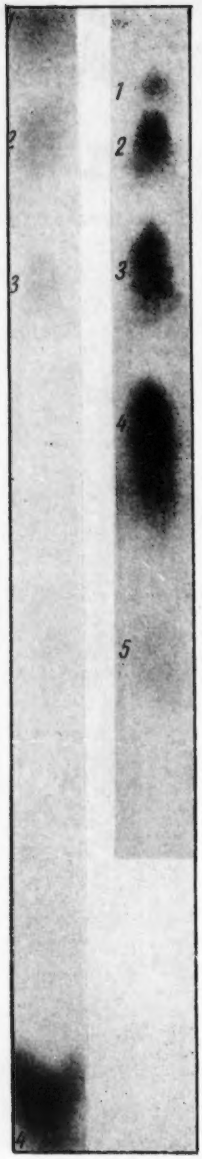

Fig. 4 Fig. 5

Fig. 4. Separation of higher fatty acids of castor oil. Acids: 1) stearic; 2) oleic; 3) linoleic; 4) ricinoleic.

Fig. 5. Separation of higher fatty acids of coconut oil. Acids: 1) stearic; 2) palmitic; 3) myristic; 4) lauric; 5) capric.

The method of reversed phase partition paper chromatography assures the separation of n-fatty acids which differ in chain length by two or even by one carbon atom (capric and pelargonic acids). The values of R_f increase when a double bond is introduced into the fatty acid molecule (stearic and oleic acids), when further double bond is introduced into the molecule, and especially when a hydroxyl group is added (ricinoleic acid).

In addition, a consideration of Figs. 1 and 2 and the table can show that shortening the chain of acids which contain 18 or more carbon atoms by two atoms produces exactly the same increase in R_f as does the introduction into such a molecule of a double bond. Thus, palmitic acid agrees with oleic acid in position, and stearic with eicosenic. This effect was noted before, in the experiments of Kaufmann [4] who gave such pairs of acids which were not separated on the chromatogram the name "critical pairs." In many plant oils acids which form "critical pairs" are found together. To determine the saturated acids in the oils in the presence of the corresponding unsaturated acids, the latter were destroyed by permanganate oxidation (Fig. 3, a and b). Mild oxidizing conditions fully protect the saturated acids from destruction. If, after the acids with double bonds have been destroyed, there is a decreased intensity of color in the spots, this can be taken as evidence for the simultaneous presence in the spots of saturated and unsaturated acids. If the given R_f value corresponds only to an unsaturated acid, then after oxidation the spot should vanish entirely.

The position of the double bond in octadecenoic acids does not affect the R_f. Therefore, for the separation of oleic and petroselinic acids which occur together in the oils of the Umbelliferae, the mixture of these acids is submitted to the oxidation procedure which has been described. When their molecules are broken and oxidized at the double bonds, the carboxyl compounds which are formed are, respectively, pelargonic and lauric acids, which are well separated on the chromatogram.

Since all three components are present in the chromatogram system which we have described, this method of separation is more effective than the methods described earlier. Thus, in the experiments of Kaufmann [4], in addition to the "critical pairs", he could not separate myristic and linoleic acids from each other, or lauric and linolenic. As the table shows, our method assures full separation of these acids. The effectiveness of the separation is also increased because acetic acid is used as the solvent instead of aqueous alcohol, and this eliminates the effect of mutual solution of the fatty acids [15]. The absence of mutual solubility makes possible the determination of components of the mixture which are present in very small amounts compared to the other components. The lower limit of sensitivity of the method is 0.03 μ Eq of acid (about 10 μg). Rare reagents or complicated apparatus are not needed to carry out the work.

Using this method we have separated the fatty acids of linseed, mustard, castor, and coconut oils (Fig. 3 4, 5), and also peanut, poppy, sunflower, and parsley oils.

As the data show, this method permits separation of the mixtures of fatty acids obtained from oils of very different compositions.

SUMMARY

We have described a reversed phase chromatographic separation of mixtures of higher fatty acids on filter paper which has been impregnated with natural rubber. The non-mobile liquid phase in the separation is a mixture of aliphatic hydrocarbons, b. p. 220-260°, and the mobile phase (solvent) is 90-99% acetic acid saturated with the same mixture. After removal of both liquid phases from the paper, the acid spots are developed as silver salts which are transformed into the dark silver sulfide. The lower limit of sensitivity of the method is 0.03 μ Eq of acid. The method permits separation of saturated fatty acid from C_{10} to C_{24} which differ in chain length by two or even by one carbon atom, and separation of the saturated fatty acids from the corresponding unsaturated and hydroxyacids, and even the separation of unsaturated fatty acids with the same number of carbon atoms, but differing in the number of double bonds. We have given the R_f values for 16 fatty acids of plant tissues. We have also described the chromatographic separation of natural mixtures of fatty acids from peanut, linseed, mustard, castor, coconut, and other plant oils.

Received January 16, 1958

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SOME PHYSICOCHEMICAL PROPERTIES OF CHLOROPLAST PROTEINS

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At the present time there has been considerable progress in the investigation of animal proteins. Proteins of plant origin have been less studied, especially the proteins of the vegetative organs of plants. Among the latter there is particular interest in chloroplast proteins, which are closely connected with the photosynthetic apparatus of the plants, which share in a number of important biochemical syntheses.

As a result of our studies on the nature of the protein systems of sugar beet chloroplasts we have shown that they are a complicated complex of a number of proteins [1]. During electrophoresis over a pH range from 6.8 to 9.5, the complexes are separated into four components which differ considerably from each other in electrophoretic mobility and in relative concentration. When the protein complexes are fractionated by acetone, ammonium sulfate, and preparative electrophoresis, a series of protein substances are separated which in electrophoresis behave like individual components [2].

The results of many investigators and our findings show that the proteins of plant vegetative organs, chloroplast proteins in particular, are present as conjugated proteins, the isolation of whose protein portion is apparently a difficult problem.

It has been shown in a number of studies that proteins isolated from plant vegetative organs contain lipids, nucleic acids, carbohydrates, and various mineral elements. Thus, Osborn [3] in fractionating spinach leaf proteins by alcohol found in the fraction which consisted chiefly of chloroplast protein 12% N and 7.4% salts, lipid substances, and 2.8% pentose. Menke [4] analyzed the chloroplast substances of spinach leaves and showed that the chloroplast protein contained 13.5% N. In its composition he found phosphorus, carbohydrates, and 7.65 or 17.8% salts. According to the data of Timm [5], spinach chloroplast protein contained 13.8% N, 4.6% salts, 0.5% P, and the cytoplasm protein contained 14.5% N and 13% salts. Osipova [6] obtained bean chloroplast protein by extraction with alcoholic alkali; such protein contained 14.8% N and 2% ash. Sisakian and Bezinger [7] isolated from sugar beet chloroplasts a lipoprotein which contained 10.9% N, 4.8-6.7% bound lipids, and 1.4% ash. Pirie [8] in his isolated nucleoproteins from tobacco leaves found 13-15% N, 2-3% P, and 10-16% carbohydrates. The proteins isolated by Wildman and Bonner [9] from spinach leaf cytoplasm contained 12.7% N, and after hydrolysis a sugar residue was observed. Kamath and Sohenie [10] isolated protein from leafy vegetables by extraction with 0.5% NaOH and determined 12.9% N and 2.17% ash in them. The chloroplast protein which we isolated [2] usually contained 10.2-13.0% N and ribonucleic acid.

The literature contains almost no information on the determination of the physicochemical properties of isolated chloroplast proteins. Osipova [6] determined the relative viscosity of chloroplast proteins from young and old leaves and obtained in 1% solutions a value of 1.70 for the first and 2.01 for the second. There are data on the ultracentrifuging of chloroplast proteins and on their molecular weight (m.w.) determined by the sedimentation constant and by other methods for the chlorophyll–protein complex. Many authors in carrying out these investigations have given data for the quantitative ratio of chlorophyll and protein in the complexes which they studied, which has permitted an estimation of the m.w. for the protein part of the complex, since the m.w. of chlorophyll is known (the m.w. of chlorophyll equals 926).

Stoll [11] isolated the green chromoprotein "chloroplastin" from 30 varieties of plants. He determined the m.w. by ultracentrifuge for the chloroplastin of aspidistra which had been treated with sodium cholate, obtaining a value of $5 \cdot 10^6$. When he studied the chloroplastin of the same plant some time later, Stoll [12] found three boundaries in the ultracentrifuge, the slowest of which corresponded to a m.w. of several million. Prise and Wyckoff [13] observed in the leaves of the cucumber plant a homogeneous substance which settled in the ultracentrifuge field with a sharp boundary at $S_{20} = 77$ Svedberg units. The solution under study was a clear green and gave positive protein reactions. The authors did not give any data on methods of obtaining the green solution and therefore we can only assume that it came from large aggregates of chlorophyll with protein and fragments of chloroplast granules. Smith [14] obtained a chlorophyll–protein complex from spinach leaves and called it "phyllochlorin"; he obtained it by treating the green mass which contained the chloroplasts with a 1-2% aqueous solution of digitonin. In the ultracentrifuge the clear green solution showed two sedimentation boundaries, of which one corresponded to m.w. on the order of 70,000, the other moved twice as fast. Later Smith [15] observed that when the spinach chlorophyll–protein complex was acted upon by a solution of detergent (Na desoxycholate, a bile salt, and digitonin) the chlorophyll was split from the protein which sedimented in the ultracentrifuge with $S_{20} = 13.5 \cdot 10^{-13}$ which corresponded to m.w. 265,000 (calculated by the Stokes formula). In the presence of Na dodecyl sulfate, chlorophyll did not split from the protein, but the complex split into smaller units. In 0.25% solution of this detergent at pH 5 to 9 it sedimented at $S_{20} = 2.6 \cdot 10^{-13}$ and in 2.5% solution there occurred a still deeper splitting and $S_{20} = 1.7 \cdot 10^{-13}$. The author considered that the value of 265,000 corresponded to the minimal value for m.w. of the protein in the native form.

Takashima [16] determined the m.w. of crystalline chlorophyll–lipoprotein complex obtained by his method from fresh leaves of Trifolium repens. In the complex two molecules of chlorophyll were found per molecule of protein. He showed that the osmotically determined m.w. of the lipoprotein in the complex was 19,200.

Wolken and Schwertz [17] determined the m.w. in seaweed chloroplasts (Euglena gracilis and Poteriochromonas stipitata) by two independent methods: on the basis of analytical data on chloroplast extracts and from geometric data obtained from the corresponding measurements of electron microphotographs of chloroplasts.

The dry chloroplasts isolated from the seaweeds were dissolved in solutions of digitonin and anions of the organic detergent ("Nacconal") and brought by buffers of pH 8.' and 9.2 to a concentration of 1.8%. According to a number of analytical results the m.w. of the pigment–macromolecular detergent complex as determined in the ultracentrifuge for the true m.w. of Euglena chloroplasts in digitonin solution was 37,000 and in "Nacconal" solution 29,000, and according to the geometrical data obtained from measurements of the electron microphotographs of the chloroplasts, 21,000. The m.w. of the chloroplasts of the other seaweed by geometric data averaged 37,000. The authors determined the concentration of chlorophyll by optical and analytical methods and showed that in their chloroplastin there was one molecule of chlorophyll per molecule of lipoprotein.

Thus we can see that the data of Wolken and Schwertz, obtained by different methods, agree well with themselves and are close to the data of Takashima.

The present study was carried out with the protein of sugar beet chloroplasts, the method of isolation of which and some data on the chemical nature were published earlier [2]. We analyzed the protein compounds precipitated from the chloroplast extract at ammonium sulfate 0.2, 0.4, and 0.7 saturation, and also the compounds called the γ-fraction.

On the basis of character of salting out by ammonium sulfate, solubility and electrophoretic mobility, the fractions of 0.2 and 0.4 were called the α- and β-chromoglobulins by us previously.

The physicochemical data obtained in our study indicate a fibrillar type of structure for the molecule of these compounds which obliges us to consider it more suitable to call them the α- and β-chromoproteins of the chloroplast.

Electrophoresis in the Tiselius apparatus showed that these fractions are electrophoretically homogeneous [2]. It is necessary to say that electrophoretic homogeneity, as is known, does not exclude ordinary polydispersion, that is, a more or less wide distribution of dimensions or m.w. of the corresponding particles in the limits

of the fraction, which form individual granules in electrophoresis.

The quantitative amounts of the protein fractions isolated by salting out with ammonium sulfate are very different. Most of the protein is in the fraction salted out at 0.2 saturation, averaging 82.8%; fraction 0.4 is 6.2% and fraction 0.7, 10.6%

In distinction to the previous method of extraction, the protein fractions obtained for the present work were freed from lipids by extraction with alcohol and ether at lower temperatures (–5°, –10°) and lyophilically dried so that in all probability there was less possibility of denaturation than could occur in the previous method of drying (by solvents). This is confirmed by the better solubility of the isolated compounds and the greater phosphorus content in fractions 0.4 and 0.7 than we had obtained earlier [2].

For a better understanding of the significance of the physicochemical constants which we obtained it was necessary to carry out a quantitative chemical analysis of the compounds which we studied. The data from these studies are given below.

EXPERIMENTAL PART

For determination of total carbohydrates we carried out separate quantitative determinations of hexoses and pentoses.

Hexoses were determined by the known colorimetric reaction of carbohydrates with the anthrone reagent [18, 19]. Measurement was carried out in a photoelectric colorimeter (FEK) at 620 mμ, with a curve for a glucose solution. For quantitative determination of pentoses we used the reaction of these with orcinol (according to Mejbaum [20]). We also used the FEK at 620 mμ with arabinose as the standard.

As Table 1 shows, considerable amounts of both hexoses and pentoses were found in all fractions. Determination of ash showed that our chloroplast protein compounds were combined with a large quantity of inorganic ions.

Nitrogen was determined by the Kjeldahl micromethod and phosphorus according to Fiske and Subbarow.

TABLE 1

Chemical Composition of Protein Compounds Isolated from Sugar-Beet Chloroplasts (in % of air dried weight)

Protein fraction	Protein (N 6.25)	Total N	Total P	Carbohydrates		Ash
				pentoses	hexoses	
0,2*	87.5	14.0	0.18	1.53	3.88	5.6
0.4*	61.2	9.8	1.70	2.17	13.49	14.0
0 7*	59.3	9.5	1.43	2.17	13,00	13.4
γ	28.8	4.6	0,28	0.60	6.00	12,3

* Saturation of $(NH_4)_2SO_4$.

This table shows that the 0.4 and 0.7 fractions contain considerable amounts of phosphorus. We showed earlier [2] that the phosphorus of the protein complexes from which these compounds were isolated occurred in the RNA which was found in combined state with the proteins.

It is important to emphasize that the compounds which we studied were subjected to reprecipitation and very long, repeated dialysis, and this makes us believe that the carbohydrates and inorganic ions are not chance contaminants, but occur in close union with the protein molecule.

As can be seen, in all the fractions there still remains some unidentified substance, which is especially plentiful in the γ-fraction.

Physicochemical Study of the Chloroplast Protein Fractions

In studying the physicochemical properties of the chloroplast proteins we determined the sedimentation coefficients (S_{20}^{w}), diffusion coefficients (D), partial specific volume (V), characteristic viscosity ([η]), and specific increment of refractive index $\frac{\Delta n}{\Delta c}$.

All the studies were carried out in 0.1 N NaCl, pH 8.0-8.5. The calculated data relate to 20° and pure water [26].

The sedimentation experiments were carried out in a large Svedberg oil ultracentrifuge at 60 thousand rpm using the optical scheme of Philpot-Swenson. Effects of concentration were absent. We used a freshly prepared 1% solution of the protein fraction for the experiment.

The diffusion coefficient was measured in a Lamm diffusion apparatus provided with an optical system connected with a scale and a thermoregulator, capable of an accuracy of 0.005°, and automatically constructed for photographing the scale. The experiments were run with a 0.3% solution.

All the other measurements were carried out with solutions of four different concentrations, from 0.5 to 2%. The concentration of the compounds studied was determined by drying to constant weight an analytical sample of the solution at 105-108°.

The partial specific volume was determined with a pycnometer, by the method of Svedberg and Pedersen [21].

Viscosity measurements were run in the ordinary Ostwald viscosimeter in a thermostat at 20°.

The specific increment of refractive index was determined with a Heber-Lev interferometer, calibrated on a KCl solution [22, 23]. The resulting values correspond approximately to the middle of the visible spectrum, that is, to the green line of mercury (545 mμ). Measurements were made at 20°.

The resulting values of V and $\frac{\Delta n}{c}$ are given in Table 2. From these we can conclude that the 0.2 fraction is a practically pure protein (although it contains an appreciable amount of carbohydrates and salts), since both V and $\frac{\Delta n}{c}$ are characteristic of an average protein [21]. The index obtained for the other fractions shows a considerably greater complexity, which is also confirmed by chemical analysis. The values of V for fractions 0.4, 0.7, and γ- are close to those for nucleic acids [24], from which, however, it does not follow that they are nucleotides.

TABLE 2

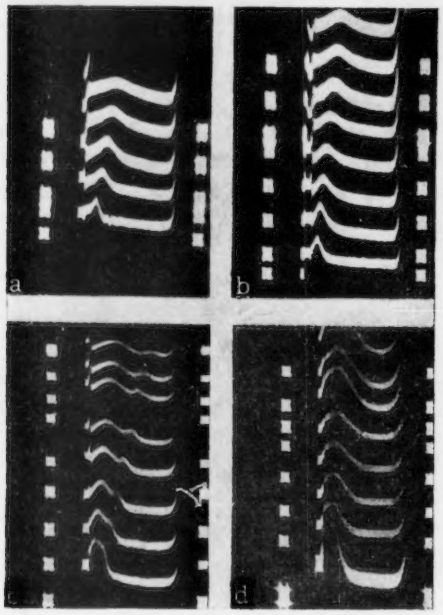
Partial Specific Volumes and Specific Increments of Refractive Index of Chloroplast Protein Compounds of the Sugar Beet

Protein fraction	V cm^3/g	$\frac{\Delta n}{\Delta c}$ cm^3/g
0.2	0.734± 0.010	0.182
0.4	0.558± 0.001	0.194
0.7	0.563± 0.012	0.057
γ	0.535± 0.010	0.034

On the basis of the far from complete results it is difficult to draw final conclusions about the nature of the three fractions. To judge by the chemical composition and values for $\frac{\Delta n}{c}$ and V, we may think that the great density of these fractions (about 2 g/cm^3) assures the inclusion of inorganic substances (in fact, for pure protein, V = 0.73 – 0.75, for pure polysaccharide, 0.62, and for pure polynucleotide, 0.55). Ions of heavy metals can be found in these inorganic inclusions; the large amount of ash in the fractions studied makes this assumption probable.

The study of the specific increment of the index of refraction shows that for the 0.7 and γ-fractions there is a considerable deflection from the norm, which correlates with the decreased protein portion of these compounds (cf. Tables 1 and 2). Since $\frac{\Delta n}{c}$ for mixtures is a additive function of the increments of the separate components, it is clear that substances with a large $\frac{\Delta n}{c}$ (that is, proteins and nucleotides) are present in fractions 0.7 and γ in small amounts. However, we should consider the possibility of a further decrease in $\frac{\Delta n}{c}$ of the strictly protein part of the complex because of binding of inorganic ions [25].

A study of the sedimentation of isolated chloroplast proteins shows that in most cases they form one boundary (figure). Fraction 0.7 is an exception which shows two sedimentation boundaries. The corresponding values for the sedimentation coefficients appear in Table 3.



Sedimentation diagram for chloroplast protein components, time of experiment 1.5-2 hours. a) 0.2 fraction; b) 0.4 fraction; c) 0.7 fraction; d) γ-fraction. Sedimentation occurred from left to right. The lower curve in each print began the experiment.

For fractions 0.2 and 0.4 we determined the diffusion coefficients. Determination of the ratio of diffusion coefficients $\frac{D_{2m}}{D_A}$ (average weight and calculated at maximum ordinate and area) showed that for fraction 0.2 it had a value of 1.10 and characterizes this protein as a comparatively monodisperse substance. For fraction 0.4 this ratio equals 1.33 which indicates a great polydispersion [26].

On the basis of the hydrodynamic characteristics we estimated the m.w. of the fractions which we studied. These results are also given in Table 3.

For a determination of m. w. under these conditions, when the sedimentation and diffusion coefficients were measured, we calculated by the Svedberg formula, with use of both diffusion coefficients. In addition we used the Svedberg formula for rigid ellipsoidal particles [21] which permits an estimate of the limiting values of m. w. with reasonable assumptions as to the coefficient of asymmetry f/f_0 from 1 to 2.

In view of the comparatively large values of the characteristic viscosity at small m. w. we could consider that the molecules of all four of the fractions had the usual inherent polymer configuration of a statistical coil. Therefore the m. w. was also calculated by the Flory formula based on a combination of $[\eta]$ with S_{20} or D [27]. For the γ-fraction an estimation of the m. w. was also made from the formula for undetermined sedimentation equilibrium in a given layer [28]. As can be seen from the data, given in Table 3, the m. w. for fraction 0.2 calculated by the different methods agree more or less satisfactorily. Also, for this fraction, the values for both diffusion coefficients are close. Hence, this fraction is comparatively monodisperse and the coiled model is satisfactory, since the correlation of m. w. calculated according to Svedberg and to Flory (M_{SD2n} and $M_{s\eta}$) is determined chiefly by the correct choice of a model and (secondarily) by the polydispersity [29].

The values of m. w. calculated by different methods for fraction 0.4 differ very greatly from one another. The disparity in values of $M_{\eta D}$ and $M_{s\eta}$ calculated by the Flory formula with the value of M_{SD} calculated by the Svedberg formula shows that the configuration of the molecule of this component approximates to a rigid rod (the pH at which the measurements were made makes a branched chain unlikely; because of effect of the charge, rapid rigidity is an "internal" property of the molecule).

For the components of fraction 0.7 we have calculated only the limiting value of m. w. at $f/f_0 = 1$ and 2. Since the m. w. here is close to the upper limit of m. w. (at $f/f_0 = 2$), we can also assume that the molecule of the chief component of this fraction is strongly stretched.

The value of m.w. for the γ-fraction shows that it is probably a complex of comparatively short polypeptides with polysaccharides [2] and with other, still unknown substances.

The physicochemical characteristics of the chloroplast proteins which we have studied indicate that their molecules have an asymmetrical structure. If we compare the values for S_{20}, w, $[\eta]$, and m.w. for all the

TABLE 3

Hydrodynamic Characteristics and Average Molecular Weight of the Chloroplast Protein Fractions

Protein fraction	$S_{20,w}$ in Svedberg units	D_{2m} in Fick units	D_A in Fick units	$[\eta]$ liter/g	$M_{SD_{2m}}$**	M_{SD_A}	M_{S_η}	$M_{\eta D_{2m}}$	$M_{\eta D_A}$	M_Z
0.2*	2.5	4.68±0.78	4.25±0.35	0.225	40 000	44 000	36 000	49 000	64 400	—
0.4*	1.56	6.39±0.20	4.77±0.10	0.13	13 400	18 000	8 500	33 000	79 000	—
0.7* (chief component)	1.93	—	—	0.057	11 000—3 800***	—	7 900	—	—	—
0.7* (small component)	4.91	—	—	—	44 000—15 400***	—	—	—	—	—
γ	0.662	—	—	0.050	—	—	1 360	—	—	3540

*$(NH_4)_2SO_4$ saturation.

**Index at M shows in what hydrodynamic parameter it is calculated.

***Calculated by the Svedberg formula for extended rigid ellipsoid rotation in the limits of variant $f/f_0 = 1$ and 2, with experimental establishment of $S_{20,w}$ and V. The large value in m. w. corresponds to $f/f_0 = 2$.

fractions studied (Table 3) we can see that there is no indication among them characteristic of proteins with molecules of globular structure. The proteins of fraction 0.2 have a very high value for $[\eta]$, considerably greater than do ordinary linear polymers with the same m.w. in neutral solvents. In other words, the relation between $[\eta]$ and m. w. in this case is like that in proteins which lack a globular structure. For the other fractions (0.4, 0.7, and γ) the disparity of m. w. and $[\eta]$ is increased even more. This suggests a rigid, rod-shaped configuration for the molecules of these fractions which is also indicated by the absence of a correlation between $S_{20,w}$ and $[\eta]$ at a clear correlation of $[\eta]$ and m. w. As is known, in rod-shaped particles $S_{20,w}$ does not depend on the their length and thus cannot characterize change in m. w. On the contrary, $[\eta]$ is strongly a function of length, that is, is a function of m. w.

Finally, it is significant that the γ-fraction, in spite the small m. w., does not dialyze. This can occur only when the molecule is fully stretched, that is, its linear dimension is great.

Thus, we can confirm that fraction 0.2 is a fibrillar protein. The other fractions are polydispersed polymers with comparatively rigid stretched molecules, similar in this sense to molecules of nucleic acids, but with smaller dimensions.

DISCUSSION OF RESULTS

Chemical analysis of the proteins extracted from sugar beet chloroplasts showed that they were not pure proteins, but a complex of conjugated proteins with a series of components in their composition: carbohydrates (both hexoses and pentoses), phosphorus, and mineral salts. It is very possible that the combination of the vegetative organ proteins with a number of other substances can be explained by the fact that the proteins of the chloroplasts and of the leaf plasma play a definite part in the various biochemical processes of the plant cell. The presence of different active substances (carbohydrates, phosphorus, salts) combined with proteins suggests a considerable variety in their biochemical potential.

The presence of heteropolysaccharides combined with one of the components of the protein system of sugar beet chloroplasts, the γ-fraction, was shown earlier [2]. The data of other authors on the carbohydrate components in chloroplast proteins was not then available to us, except for the evidence of Menke [4] on the presence of a carbohydrate-containing fraction in spinach chloroplast substances and the indirect evidence of Osborn [3] who showed 2.5% pentose in the fraction which we isolated from spinach leaves and which could be related to the chloroplasts from the chemical analysis.

The large amount of ash found in the chloroplast proteins agrees with the results of a number of authors. It has been shown that most of the ash of plant cells is found in the chloroplast [30-33] and sometimes reaches 16.9%. The elements of the ash are very diverse and consist of a number of ions [31, 32]: Fe, Ca, Mg, Cu, P, Na, Cl, SO_4. There is preferential localization of Mg, Cu, Zn, and Fe in the chloroplasts [33]. It has been shown that most of the Fe of the chloroplasts is bound with the protein fraction [34] and most of the Cu is bound to organic compounds [31]. A large part of the salts has been found by a number of authors in the chloroplast protein composition [3-5].

The m. w. value of 40,000 determined by us for fraction 0.2 which is the chief mass of chloroplast protein, is close to the value of Wolken and Schwertz [17] and also of Takashima [16]. The variations in the results for the m. w. of the chlorophyll–protein complex obtained by different authors and the large values may be explained by the heterogeneity of this complex and the presence in it of fragments of chloroplast granules and plasma proteins which could be adsorbed on the complex, since the earlier methods of isolation were not sufficiently perfected [35]. Also, the determinations were carried out in solutions of detergents which can themselves form micelles in water with high m. w. (75,000-400,000) [36], which are able to absorb proteins, thus producing distorted results.

On the other hand, it may be possible that the detergents, reacting with the proteins, block not only the ionizing groups of the protein molecules, but also can combine with them by their non-polar carbohydrate groups [37] which react with the non-polar groups of the protein. There could then occur splitting of lipids from the protein and also their combination with the non-polar protein groups. As a result of such competition for the non-polar protein groups between the lipids and the detergent, the lipid could be split from the protein as large protein--lipid complexes, which would then split into smaller units. It is very possible that these effects occurred in the experiments of Smith who obtained different values of m. w. in successive treatments of the chlorophyll–protein complex by detergents [15]. Thus it seems likely that the protein with m. w. 265,000 is a protein–lipid complex which on further treatment with detergent would split to form protein molecules free from lipids and sedimenting with $S_{20} = 2.6$ or $1.7 \cdot 10^{-13}$.

The latter value agrees with the sedimentation constant obtained in our investigation (Table 3), but it is necessary to remember that the protein which we isolated was freed from substances of a lipid nature during its purification [2], which probably excludes the possibility of complex formation in large aggregates such as can occur in the presence of lipids.

It is especially interesting, as it seems to us, that in this study we observed a definite lack of globulin form and a stretched molecule for the chloroplast proteins. This may have great biological significance. Complexes with a much greater number of chlorophyll molecules may be formed with very asymmetric protein molecules than with molecules which have a globular form, and hence also, a minimum surface. Thus we can expect a considerable increase in active surface of the chlorophyll–protein complex with the participation of which solar energy is transformed into chemical energy.

SUMMARY

We have determined quantitatively the chemical composition of beet sugar chloroplast proteins. We have shown that they are complex conjugated proteins in whose composition are found carbohydrates and mineral salts in addition to nucleic acids.

For the fractions obtained by salting out with ammonium sulfate at 0.2, 0.4, 0.7 saturation and for the γ-fraction we have determined the sedimentation constants, the specific partial volumes, the specific increment of refraction, the characteristic viscosity; for fractions 0.2 and 0.4, also the diffusion coefficients.

For all the protein fractions we have calculated the average m. w. (M). Fraction 0.2 is comparatively monodisperse, M = 40,000. The other fractions are polydisperse. The most probable m. w. (M_{SD_2m} or $M_{s\eta}$) of these fractions is the following: for fraction 0.4 m. w. = 850-13,400; for fraction 0.7 M = 7900 (chief component); for γ, m. w. = 1360 or M = 3540.

Comparison of the characteristic viscosities, sedimentation coefficients, and m. w. permits us to assume that the molecules of all the chloroplast protein compounds are not globular but are either strongly unfolded statistical coils, or very rigid rods.

The authors express thanks to Prof. S. E. Bresler for making possible the experiments on determing the sedimentation constants and diffusion coefficients under his guidance and for his interest in the work.

Received January 20, 1958.

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THE EFFECT OF POTASSIUM IONS ON PHOSPHORYLATION PROCESSES IN SACCHAROMYCES CEREVISIAE

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Lynen [1] and Lynen and Koenigsberger [2, 3] showed that under anaerobic conditions the inorganic phosphorus content of yeast is higher than under aerobic conditions and assumed that this depended on different rates of phosphorylation with the same rates of dephosphorylation. This attempt to explain the Pasteur effect met objections from Ochoa and Stern [4], Stickland [5], and Warburg [6]. Up to now, the most acceptable theory has been that of Engel'gardt and Sakov, according to which oxygen acts on the reaction between adenosinetriphosphate (ATP)* and fructose-6-phosphate [7]. However, for a definite type of yeast a stationary content of inorganic phosphate evidently corresponds to a degree of aerobiosis. Krebs [8] considered that the mechanism which regulated the relation of respiration and fermentation in the cell was complex, and that ions of alkali metals, and especially of K^+, played a part in this regulation (Chance et al. [9], Krebs et al. [10], Rothstein [11], Rothstein and Demis [12]), and the K content in yeast was considerable.

Since K^+ raises the respiratory coefficient of yeast (and this is reflected in going over to anaerobic metabolism), and since anaerobic conditions correspond to a higher level of inorganic phosphate [1], it seemed interesting to us to determine whether K^+ raised the content of inorganic P in yeast, and the possible mechanism of this effect.

We give below our results from a study of the phosphorylation process in yeast under aerobic and anaerobic conditions and the effect of ionic composition of the medium on these.

METHODS

Yeast. We always used freshly pressed bakers'yeast, the dry residue of which varied between 28-31% dry weight and which contained 8-11 mg P, 0.009-0.061 mmole Na, and 0.280-0.435 mmole K per 1 g dry weight.

A series of experiments was carried out with pure cultures of Saccharomyces cerevisiae, Strain R-12, which showed that all of the processes which we studied were the same as those in bakers' yeast.

To prevent large variations in phosphate content, at the beginning of the experiments the yeast was freed from endogenous substrates and part of the ionic content by aeration of the yeast suspension in water or more often in 0.5 N NaCl. This lowered the content of intracellular K^+ so that the addition of K^+ to the medium became more effective.

Incubation of the yeast. Incubation of a 5-10% yeast suspension was carried out either in pure water or in an 0.1 N solution of NaCl, KCl, or RbCl. Preliminary experiments showed that 0.1 N salt concentrations were more favorable osmotically, and that the action of K^+ was the same within the limits of a wide range of concentrations from 0.1 N to 0.01 N (the total concentration of KCl and NaCl was always 0.1 N). The

* Abbreviations: ADP – adenosinediphosphate; ATP – adenosinetriphosphate; TCA – trichloroacetic acid; DNP – dinitrophenol; P_i – inorganic phosphate; P_t – total phosphate; P_γ – labile phosphate; P_m – metaphosphate; P_{res} – residual phosphate.

incubation temperature was usually 24°, but a number of measurements were made at temperatures from 12 to 38°. The suspension was placed in a round-bottomed flask and through a tube which reached to the bottom of the vessel was passed air, oxygen, or nitrogen, purified by washing with alkaline pyrogallol. In picking up the samples and adding the different solutions we used a pipet with a wide mouth, which permitted transferring liquids within 2 seconds. The vessel with the suspension was put in a thermostatically controlled water bath or remained at room temperature.

As a substrate we used glucose with a final concentration of 5%.

Extraction and fractionation of the phosphorylated yeast compounds. We stopped the incubation by two processes.

1. A sample was transferred to a trichloroacetic acid (TCA) solution [1, 13] whose final concentration was 6%. Extraction lasted 30 min at temperatures which did not exceed 23°.

It was shown that at temperatures between 2 and 26°, TCA extracted the same amount of phosphorylated compounds, while at temperatures above 26° their decomposition apparently began, so that we obtained unusually high values for the content of inorganic phosphate (P_i). The phosphorus extracted by TCA was hereafter called acid-soluble phosphorus.

2. A sample was centrifuged rapidly and the precipitate was washed with alcohol and ether according to the recommendation of Schmidt et al. [14]; then the phosphoric esters were removed with cold water. This process did not permit us to get a phosphorylation curve in a short time interval, but on the other hand, we could remove by it all the components extracted by TCA and even compounds of the type of metaphosphates. This phosphorus was later called water-soluble phosphorus.

After such treatment, we carried out supplementary extraction by alkali at pH 9 or 12 (alkali-soluble phosphorus). In the extracts and also in the residues after extraction we carried out determinations of P_i, total phosphorus (P_t) after mineralization according to Hanes and Isherwood [16], labile phosphorus (P_7) after 7 min hydrolysis in N HCl, and finally, "metaphosphate" precipitated by barium acetate at pH 4.2 [14]. "Metaphosphate" here means all the fractions precipitated by this reagent; actually, they are probably not homogeneous in composition and contain linear polyphosphates and cyclic metaphosphates with different degrees of polymerization; the content of high-energy bonds varies between 85 and 90% of the P which they contain [15a, b].

Determination of phosphorus. Phosphorus was determined by several modifications of the method of Martin and Doty [17] or of Hauge, Cheldelin, and King [18]. For colorimetric measurements we used the Spekker photocolorimeter and cuvettes with a 1cm thick layer and a dark red filter. Within the limits of 0.5 to 12 μg P the differences in parallel experiments did not exceed ±2%; 0.1 μg P still gave a measurable color.

Experiments using P^{32}. Phosphate containing P^{32} was added to a culture medium which contained glucose, NH_4^+, inorganic ions, and growth substances. In several cases the incubation medium contained only glucose and Na or K ions. In this case, at the end of the incubation, the suspension was quickly centrifuged and the yeast was passed through a layer of inert silicon oil, and, separated from the culture liquid, was denatured with TCA. The reason for this treatment was described by Werkheiser and Bartley [19].

Measurement of the radioactivity was carried out on an aluminum base or in a bronze container with a Superscaler apparatus. Average values were calculated for three one-minute measurements.

Inhibitors. For blocking phosphorylation we used 0.1 M HCN [1] or 0.75 M urethane [5]. We used urethane most often, since this compound is non-volatile and non-toxic. We also used the action of DNP [12] in concentrations fron $2.5 \cdot 10^{-4}$ to $2.5 \cdot 10^{-3}$ M. In view of the studies of Stickland [5, 13] which showed that arsenic salts sharply inhibited respiration without touching phosphorylation, we also studied the effect of these salts in concentrations of 10^{-4} M.

EXPERIMENTAL PART

Phosphorylation Processes and Dephosphorylation Processes in Yeast: Effect of Substrate and Oxygen

Change in content of inorganic phosphorus. The cell wall of yeast effectively protects the interior of the cell from osmotic effects of the outer environment, and therefore we could confirm the basic results on phosphorylation in yeast in media which did not contain alkali metal ions.

Figure 1 shows the change in P content in yeast during incubation in water. At moment A, aeration of sample I was begun and glucose was added to sample II under anaerobic conditions. At the moment B, glucose was added to sample I, and air was introduced into sample II. We measured the rate of phosphorylation in a number of such experiments; we showed that this proceeds in accord with the equation of a first order reaction. This relation is maintained in the first 40 sec after adding substrate under aerobic conditions, between the first and third minute under anaerobic conditions, and, without substrate, during the first 90 sec after adding air. Table 1 shows the values for the rate constant of these processes at different temperatures.

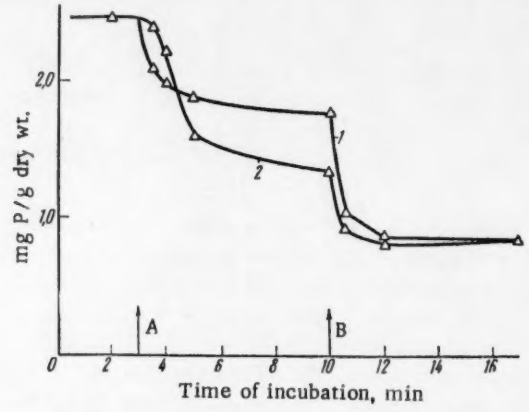

Fig. 1. Effect of glucose and air on the inorganic phosphorus of yeast; 1, 2) parallel samples; A – beginning aeration of sample 1; to sample 2 was added glucose. B – beginning aeration of sample 2; to sample 1 was added glucose

TABLE 1

Effect of Temperature on Rate Constant of Phosphorylation in Yeast at Different Temperature and Conditions of Incubation (constants expressed in sec^{-1}; figures in parentheses – number of determinations)

Reaction conditions	12°	20°	24°	36°	Temperature coefficient
Aerobic, 40 sec after adding substrate	$1.0 \cdot 10^{-2}$ (2)	$1.7 \cdot 10^{-2}$ (4)	$2.2 \cdot 10^{-2}$ (6)	$5.1 \cdot 10^{-2}$ (2)	2.0
Anaerobic, between 1 and 3 min after adding substrate	$1.8 \cdot 10^{-3}$ (2)	$3.2 \cdot 10^{-3}$ (4)	$4.0 \cdot 10^{-3}$ (6)	$9.0 \cdot 10^{-3}$ (2)	1.9
Without substrate, 90 sec after adding air	$1.2 \cdot 10^{-3}$ (2)	$2.1 \cdot 10^{-3}$ (2)	$2.9 \cdot 10^{-3}$ (4)	$6.4 \cdot 10^{-3}$ (2)	2.0

Table 1 shows that at the temperatures ordinarily used for incubating yeast, phosphorylation under aerobic conditions occurs 5 times more intensely than under anaerobic conditions. At the expiration of these periods dephosphorylation begins in the yeast suspensions and this soon completely balances the phosphorylation.

The temperature coefficient (Q_{10}) of phosphorylation in all cases was ~2.0.

In Fig. 1 it is possible to see the slow decrease in P content under anaerobic conditions, which can be explained by the presence of some intermediate process which precedes phosphorylation as such.

Distribution of phosphate in the TCA extract. Since under these experimental conditions we do not find loss of P_i from the yeast cell into the medium, it is important to explain into which fractions the P_i is distributed.

The results of the determination of content of P_i and P_t of the TCA extracts in the first seconds after adding substrate are given in Fig. 2.

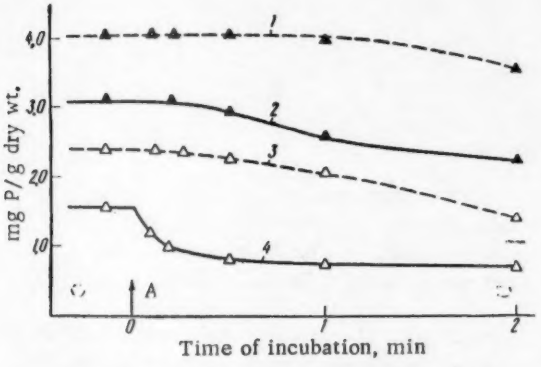

Fig. 2. Effect of aeration and glucose on the content of inorganic and total acid-soluble phosphorus in yeast: 1, 2) P_t; 3, 4) P_i; 1, 3) anaerobic samples; 2, 4) aerobic samples; A – addition of glucose in all samples.

TABLE 2

Change in Content of the Fraction ($P_t - P_i$) of Acid Soluble Phosphorus in the Presence of Glucose (in mg P/g dry weight. Figure in parentheses is experiment number.

Time after adding glucose, in min	Aerobic	Anaerobic
0	1,38 (10)	1,58 (8)
0,5	2,02 (8)	1,74 (6)
1	1,57 (8)	2,01 (6)
5	1,35 (8)	1,51 (6)
10	1,34 (10)	1,53 (8)

The general course of the curve for P_t deserves attention. In the experiments of great length the curve for P_t is almost parallel to the curve for P_i. This agrees with the results of McFarlane [20] and Lynen [1] but contradicts the data of Stickland [5] and shows that the amount of acid-soluble phosphate less the P_i does not change and that P_i enters the composition of some compound which is not removed by TCA. However, in the first seconds after adding the substrate the content of P_t is constant and this indicates that in phosphorylation of glucose there is first formed an ester extracted by TCA which then splits and the phosphate goes over to the component which is not removed by TCA. The change in the fraction $P_t - P_i$ in the TCA extract during incubation in pure water is shown in Table 2.

Retardation of the P_t curve is noted compared to the results of measuring samples without substrate, when the curves of P_i and P_t have already begun to parallel each other and when there is direct inclusion of P_i in some component not removed by TCA.

Dephosphorylation processes. The P_i content is not changed until the concentration of endogenous or exogenous substrate has fallen to a definite value. During this time the rates of phosphorylation and dephosphorylation are equal and since aerobic phosphorylation is more intense than anaerobic, the dephosphorylation rate in this phase should under aerobic conditions be higher than under anaerobic. Therefore we would expect that after blocking of the phosphorylation we would find it possible to determine directly the difference between rates of dephosphorylation under different conditions.

Figure 3 shows the changes in content of P_i and P_t in the TCA extract after adding urethane as an inhibitor of phosphorylation [5].

It is evident that the formation of P_i proceeds aerobically and anaerobically at the same rate, and at first we find a slight slowing, the reason for which is not clear. This reason for the identity of rates ($K = 6\text{-}8 \cdot 10^{-3}$ sec) is understood when we consider the change in acid solubility of P. Addition of urethane frees the phosphate esters removed by TCA from their bonds with structural (?) components, after which slow splitting of P_i occurs. This is certainly the explanation. Stickland [5] has an explanation which concerns the results obtained with urethane.

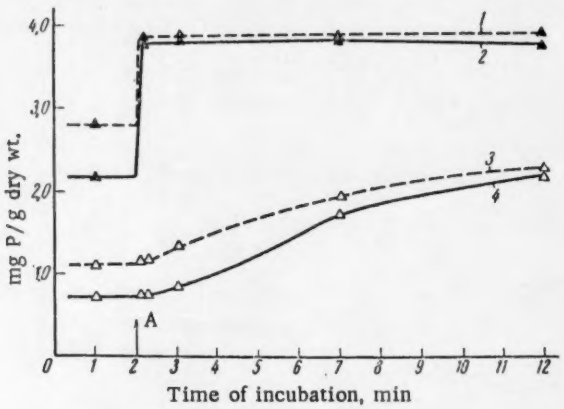

Fig. 3. Effect of urethane on the content of inorganic and total acid-soluble phosphorus in yeast. 1, 2) P_t; 3,4) P_i; 1, 3) anaerobic samples; 2, 4) aerobic samples. A – addition of urethane in all the samples (final concentration 0.75 M).

General distribution of phosphate during phosphorylation and dephosphorylation. Since during incubation of a yeast suspension in a medium which does not contain phosphate, no change in total phosphate occurs, the change in acid-soluble P_i and P_t is reflected in the alkali-soluble and residual P.

Aeration and addition of glucose in this case act in qualitatively the same way, while the addition of urethane has a reverse effect. Aside from the change in P_i content, the most obvious is the contrary direction of change in content of alkali-soluble "metaphosphate", which shows the relation between both process (Tables 3-6). The change in acid-soluble "metaphosphate" is an artifact. In all our experiments we find an increased content of labile phosphate when the substrate is fermented and its decrease after addition of urethane.

Distribution of P^{32}. The assumption of the inclusion of P_i in the "metaphosphate" was confirmed by experiments in which we used P^{32}. After a one-day incubation in a medium which contained glucose, NH_4^+, and K_2HPO_4 labeled with P^{32}, we obtained a different level of inclusion in the different fractions of a TCA extract in the presence of glucose and urethane.

While the specific radioactivity (SA) of the P_i (1130 counts/sec/μg P) after one-day culturing is the same as the SA of the medium, the activity of the " residual" phosphate (P_{res}) is only half this value. The SA of P_i ($P_t - P_i$), equal to 980 counts/sec/μg P, is not changed during the experiment, but the SA of P_{res} rises after addition of glucose from 550 to 575 counts/sec/μg P; however, under the influence of urethane this falls to the starting level. So we can assume that that portion of the P_{res} which shares in active phosphorus metabolism with the other components is in equilibrium with them and comprises about 50% of the fraction not extracted by TCA.

Figure 4 shows the change in SA of the separate fractions of normal yeast during incubation in a medium which contains labeled phosphorus. Up to point A there was aerobic fermentation without substrate, during which the SA of P_{res} increased. However, the SA of ($P_t - P_i$) remained unchanged, which again confirmed the idea of the direct inclusion of P_i in the components which were not removed, during the course of endogenous fermentation. After the addition of glucose we found an increased SA in all fractions; after the addition of urethane, a decreased SA in the P_i fraction because of rapid decomposition of inactive P_{res}.

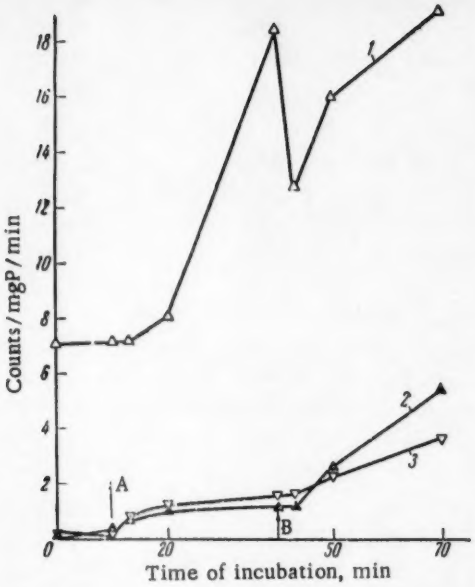

Fig. 4. Specific activity of yeast phosphorus fractions. 1) P_i; 2) P_{res}; 3) fraction $P_t - P_i$ of the acid-soluble phosphate. A – Addition of 5% glucose; B – addition of urethane to a concentration of 0.75 M. Before the experiment the suspension was kept with added P^{32} at 2° for 20 min.

TABLE 3

Change in Content of Inorganic Phosphate in Yeast in the Absence of a Substrate (in mg P/g dry weight)

Time after beginning aeration, in min	Medium	
	0.1 N NaCl	0.1 N KCl
0	2.06	2.06
1	1.57	1.61
2	1.38	1.48
5	1.27	1.39
10	1.21	1.34

In concluding this section we should note that the introduction of air into the anaerobic suspension and the addition of glucose have the same effect qualitatively on the separation of the phosphate into the individual fractions: namely, a decrease in P_i content, accompanied by an almost parallel decrease in the amount of acid-soluble P, which equals the rise in alkali-soluble phosphorus, expecially the "metaphosphate." The addition of urethane not only blocks completely the phosphorylation, but also the splitting of the phosphate esters which are removed by TCA, and thus also the elimination of the difference in rate of aerobic and anaerobic dephosphorylation.

Starting from these results, we can turn to a study of the effect of alkali metal ions on fermentation.

Effect of K^+ on Phosphorylation and Dephosphorylation

Process of phosphorylation in the absence of a substrate

As Table 3 shows, with fermentation of an endogenous substrate, which is not a polysaccharide [21], there is a decrease in P_i.

The change in residual fraction after 10-min action of air is shown in Table 4.

TABLE 4

Change in Content of Phosphate Fractions in Yeast in the Absence of Substrate 10 Min after Beginning Aeration (in mg P/g dry weight)

Medium	Fraction ($P_t - P_i$) of acid-soluble P	Fraction ($P_t - P_i$) of water-soluble P	Total alkali-soluble P	Water-soluble metaphosphate	Easily hydrolyzed acid-soluble P	Easily hydrolyzed water-soluble P
			Initial content			
0.1 *N* NaCl	1.61	2.24	1.76	0.78	0.98	0.80
0.1 *N* KCl	1.59	2.28	1.83	0.80	0.99	0.83
			After 10 min			
0.1 *N* NaCl	1.40	2.44	1.84	0.92	1.22	0.88
0.1 *N* KCl	1.40	2.47	2.00	1.14	1.22	1.23

The results in Table 4 show a difference between the rate constants for the phosphorylation of endogenous substrate in NaCl ($K = 3.0 - 3.7 \cdot 10^{-3}$/sec) and in KCl ($K = 2.6 - 3.0 \cdot 10^{-3}$/sec). The values given are the averages of 18 measurements. The difference in values in the stationary state were submitted to statistical analysis; it was shown that these values correspond to a probability of $p \ll 0.01$; this indicates a high significance in the differences.

In endogenous phosphorylation the "metaphosphate" is increased in a medium with K^+ more than in a medium with Na^+. We can assume that K^+ permits formation of "metaphosphate" and even high-energy bonds, but the cost of P_i is less than in a medium with Na^+.

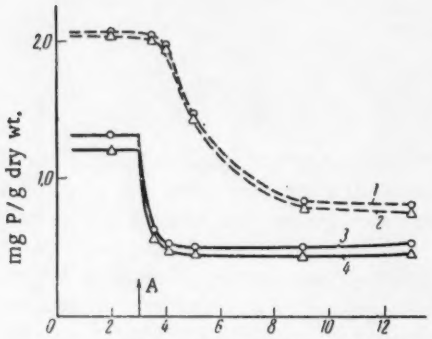

Fig. 5. Effect of glucose on content of inorganic phosphorus in yeast in connection with the ionic composition of the medium. 1) 0.1 m KCl anaerobic; 2) 0.1 M NaCl anaerobic; 3) 0.1 M KCl aerobic; 4) 0.1 m NaCl aerobic. A – Addition of 5% glucose.

Glucose as a substrate

When we used glucose as a substrate at a final concentration of 5% we found the same changes as in endogenous fermentation (aerobic and anaerobic). Figure 5 shows the changes in P_i content in aerobic and anaerobic incubation of yeast in 0.1 N NaCl and in 0.1 N KCl.

The difference in initial level of P_i in the aerobic experiments can be explained by the difference in rate of endogenous phosphorylation which we mentioned in the previous part. Phosphorylation in the presence of substrate goes with the same rate in both media, but the stationary level of P_i is higher in the presence of KCl from which we can conclude that in this case dephosphorylation is more intense. Statistical analysis of the values of P_i in the stationary state showed that the difference induced by the presence of K^+ was significant in the aerobic samples, but in the anaerobic it corresponded as an average of 28 measurements only to $p = 0.02$.

In Table 5 we give the change in content of P_i in the other fractions.

Here we can note that there is most of all a considerable increase in amount of "metaphosphate" in the presence of K^+ in the aerobic samples, while the content of phosphate esters removed by TCA is somewhat lowered. Anaerobic samples do not show a difference in formation of "metaphosphate" in KCl and NaCl. Probably in the fermentation of glucose under the action of K^+ the separation of P_i begins from some structural component and this leads to an increased stationary content of P_i. In addition, we can assume that here also factors act on the equilibrium between metaphosphate and orthophosphate.

Effect of inhibitors on the phosphorylation process

In order to obtain a more detailed explanation of the mechanism of action of K^+ on the phosphorylation process, we compared the effects of some inhibitors on these processes in media with NaCl and KCl.

Urethane. With urethane as the inhibitor we found agreement with the expected reverse change in phosphorylation compared with that which occurred after addition of the substrate. Table 6 shows the content of the separate fractions in an aerobic experiment 20 min after adding urethane, when most of the fractions have reached a stationary level.

Measurement under anaerobic conditions was carried out in a completely analogous way. As Table 6 shows, all the yeast fractions come to equilibrium and the level which is reached can be varied to one side or the other of the initial value.

The behavior of the water-soluble fraction, obtained after preliminary extraction with TCA, deserves special attention. Under the influence of urethane the content of this fraction falls by almost half. The activity of the portion which splits off in the case of a culture medium with labeled P^{32} is in equilibrium with

TABLE 5

Effect of Glucose on the Phosphorus Fractions of Yeast in Connection with the Ionic State of the Medium (in mg P/g dry weight)

Fraction	Initial content		10 min after addition of glucose		Number of measurements
	0.1 *N* NaCl	0.1 *N* KCl	0.1 *N* NaCl	0.1 *N* KCl	
Aerobic					
P_i	1.29	1.40	0.55	0.65	28
Fraction ($P_t - P_i$) of acid-soluble P	1.40	1.40	1.42	1.34	22
Fraction ($P_t - P_i$) of water-soluble P	2.44	2.47	3.54	3.50	10
Total alkali-soluble P	1.84	2.00	2.28	2.59	6
Water-soluble metaphosphate	0.92	1.14	1.11	1.31	10
Easily hydrolyzed acid-soluble P	1.22	1.22	1.21	1.23	6
Easily hydrolyzed water-soluble P	0.88	1.23	1.41	1.69	10
Anaerobic					
P_i	2.40	2.42	1.05	1.08	16
Fraction ($P_t - P_i$) of acid-soluble P	1.61	1.59	1.62	1.55	12
Fraction ($P_t - P_i$) of water-soluble P	2.24	2.28	3.48	3.46	4
Total alkali-soluble P	1.76	1.83	—	—	—
Water-soluble metaphosphate	0.78	0.80	1.09	1.09	4
Easily hydrolyzed acid-soluble P	0.98	0.99	0.97	1.01	4
Easily hydrolyzed water-soluble P	0.80	0.83	1.07	1.07	4

TABLE 6

Effect of Urethane on the Phosphorus Fractions in Yeast (in mg P/g dry weight)

Fraction	Initial concentration		20 min after adding urethane		Number of measurements
	0.1 *N* NaCl	0.1 *N* KCl	0.1 *N* NaCl	0.1 *N* KCl	
P_i	0.55	0.65	1.72	1.78	12
Fraction ($P_t - P_i$) of acid-soluble P	1.42	1.34	1.61	1.63	12
Fraction ($P_t - P_i$) of water-soluble P	3.54	3.50	2.68	2.70	10
Total alkali-soluble P	2.28	2.59	1.95	1.92	6
Water soluble-metaphosphate	1.11	1.31	0.94	1.18	10
Easily hydrolyzed acid-soluble P	1.21	1.33	1.11	1.16	6
Easily hydrolyzed water-soluble P	1.41	1.69	1.02	1.14	10

the incubation medium, while almost half the residue consists of P_7. This shows that urethane causes only partial splitting of high-energy bonds which are present in the cells.

DNP. As Lynen [3] and other authors showed, DNP has an effect on the Pasteur reaction. Since K^+ acts qualitatively in the same direction, we studied the effect of DNP on phosphorylation and dephosphorylation under aerobic and anaerobic conditions, without substrate and with glucose, in media with KCl and with NaCl. Within the limits of the DNP concentrations studied, it fully suppressed endogenous phosphorylation; after addition of substrate the differences between the stationary levels of P_i in media with KCl and with NaCl under aerobic and anaerobic conditions were maintained. In the presence of the inhibitor, the content of P_i on the average was 20-35% higher aerobically and 10-15% higher anaerobically than without it. The aerobic rate of phosphorylation under the influence of DNP fell by one degree; the anaerobic remained practically unchanged. The rate of dephosphorylation after addition of urethane was the same as without DNP.

In spite of the similarity in action of K^+ and DNP with respect to raising the respiratory coefficient and increasing the level of P_i, the points at which their action is applied evidently differ. While K^+ without a

substrate raised the formation of "metaphosphate", DNP suppressed its formation under the same conditions.

Arsenic salts. As Stickland showed [5], arsenic salts in concentrations of about 10^{-4} M caused a reduction in the respiration of yeast of about 70%, during which phosphorylation proceeded almost unchanged, and, on the contrary, the stationary level of P_i became lower than in the absence of the arsenic salts. He assumed that the sensitivity of yeast respiration to arsenic applied to all phosphorylation in the cell. We studied the possibility of adding K^+ to remove the effect of arsenic salts which decreased the constant level of P_i. The experiments showed that the difference between content of P_i in media with KCl and NaCl was maintained even in the presence of arsenic salts.

TABLE 7

Effect of Dinitrophenol and Arsenate on Content of Inorganic Phosphate in Connection with Addition of Glucose (in mg P/g dry weight)

Time after adding glucose in min	Without inhibitor		Number of measurements	$2.5 \cdot 10^{-3}$ M DNP		Number of measurements	10^{-4} sodium arsenate		Number of measurements
	0.1 *N* NaCl	0.1 *N* KCl		0.1 *N* NaCl	0.1 *N* KCl		0.1 *N* NaCl	0.1 *N* KCl	
0	1.29	1 40	22	1.60	1.61	5	1.37	1.51	2
1	0.67	0.75	10	1.01	1.04	2	0.65	0.74	2
10	0.55	0.65	22	0 71	0.80	5	0.49	0.58	2

Thus, here, as in the case of DNP, K^+ acts differently from the arsenic salts. In Table 7 we give the data concerning the content of P_i under aerobic conditions in the presence of DNP and arsenate.

Cultures in a medium which contains cyanide. In order to show the role of K^+ in phosphorylations which are related to the cytochrome system, we cultured yeast according to the recommendations of Pett [22] in media which contained about 10^{-3} M HCN. This inhibited the action of the cytochrome system, and these functions were taken on by the flavins. In two of the three experiments which we ran, we found no effect of K^+ under these conditions, while in the third experiment the effect of K^+ was the same as in the ordinary media. The data from this series of experiments are unreliable, since the decreased P_i content after adding glucose was slight, although the respiration of the yeast remained almost normal; therefore it is impossible to draw any conclusions from these experiments.

Effect of Rb^+ on Phosphorylation Processes

As a number of authors have shown in studies of the action of alkali metal ions on the metabolic processes of microorganisms (for example, Macleod and Snell [23]), Rb^+ can to a definite extent replace K^+ in its metabolic function; the penetration of K^+ and Rb^+ through cell walls is similar. Table 8 shows the content of acid-soluble and water-soluble P_i after incubation in the presence of 0.1 N NaCl, KCl, and RbCl (average of two experiments).

TABLE 8

Effect of Na^+, K^+, and Rb^+ on Content of Water-soluble Inorganic Phosphate and Acid-soluble Metaphosphate in Yeast (in mg P/g dry weight)

Experimental conditions	0.1 *N* NaCl		0.1 *N* KCl		0.1 *N* RbCl	
	P_i	P_m	P_i	P_m	P_i	P_m
10 min anaerobically	2.32	0.74	2.30	0.78	2.31	0.74
10 " aerobically	1.53	0.92	1.62	1.10	1.62	1.06
3 " (glucose)	0.74	1.10	0.81	1.28	0.80	1.27
10 " "	0.73	1.10	0.80	1.30	0.80	1.28

The results conclusively show the similar action of Rb^+ and K^+ on the fermentation of endogenous and exogenous substrates. Evidently the action of K^+ in the phosphorylation processes cannot be considered fully specific.

DISCUSSION OF RESULTS

Relation between Processes of Phosphorylation and Dephosphorylation

From kinetic data concerning the rates of phosphorylation and dephosphorylation it follows that the stationary level of P_i in yeast depends on the ratio of rate constants of both processes. From the equation for rate constants

$$K_e = \frac{[AP]}{[A][P]}$$

where A is the substrate of phosphorylation, P orthophosphate, and AP the resulting ester, it is evident that there are two possible cases for raising the equilibrium concentration of P_i.

On the one hand, this will happen by raising the concentrations of all the components without changing K_e, which seems unlikely and does not correspond to the experimental results (if we double the concentration of P, then the concentration of AP should increase fourfold). On the other hand, the same result will be found by changing the equilibrium constants by the equation:

$$^1/_2(P) + {}^1/_2(A) + (AP) = K$$

This equation means that in the formation of one gram molecule of AP there is used one gram molecule of the reaction components; in order to express the constancy of the total amount of reaction components, we must multiply their concentrations by a factor of 0.5. This assumption is more acceptable and it is qualitatively confirmed by the relation of rate constants of phosphorylation and dephosphorylation. The aerobic rate constant of phosphorylation is higher than of dephosphorylation; the reverse relation holds for the anaerobic constant so that under anaerobic conditions there is a higher level of P_i. From the point of view of energetics, however, it should not be forgotten that phosphorus metabolism can occur in different ways and that the requirement of substrate under aerobic conditions is lower than under anaerobic. We cannot therefore exclude the possibility that under different conditions of incubation the energy of metabolism is differently used and that the value of the stationary level does not correspond to the energetics of the metabolic process. The question always remains unsettled, how much does the rate constant of phosphorylation depend on changing conditions of permeability to glucose which was shown by the results of Burger [24]. Taking this into account, we must estimate the possible action of K^+ on the processes of phosphorylation and dephosphorylation. The clearest and best determined effect of K^+ in yeast maintained in a medium which lacks this ion is to increase the stationary level of P_i as compared to yeast incubated in a medium without K^+. When, in the absence of added substrate, there is phosphorylation only of endogenous substrate, the rate constant of phosphorylation in a medium with K^+ is lower than in a medium with NaCl, while the constant for dephosphorylation is the same in both cases. The difference in the stationary level of P_i occurs under aerobic conditions even in the presence of substrate, but the difference in the rate of phosphorylation is not found. The difference in P_i content with NaCl and KCl media under anaerobic conditions can be explained by the assumption that in the presence of any exogenous substrate the dephosphorylation constant in all circumstances is higher for media with KCl than for media with NaCl. We can assume that the rate of endogenous phosphorylation does not affect the stationary level of P_i in the presence of exogenous substrate and that the observed difference depends only on the difference in rate of dephosphorylation. This idea is confirmed by the fact that the difference in stationary level of P_i is maintained even in the presence of DNP, when endogenous phosphorylation is entirely suppressed and when, because of this the initial content of P_i is the same in media with NaCl and KCl.

Mechanism of Action of K^+

As to the mechanism of action of K^+, the following are some of the possibilities.

1. The effect of K^+ on the physicochemical properties of the cell wall, for example, changing its bioelectric potential and permeability (but we cannot thus explain the difference on endogenous metabolism).

2. Lowering the amount of energy required for active transfer, or for keeping the concentration gradient.

3. Effect on the enzymatic processes of the surface or internal medium of the cell.

The last explanation is the most acceptable. We must account also for the activation of ATP-ase by K ions (experiments in this direction are continuing) and their effect on formation of metaphosphates. As was shown by Schmidt [14], Belozerskii [25], and other authors, metaphosphate can serve as a source for chemical energy in the cell. In distinction from Schmidt, we have carried out experiments in media without phosphate, and have thus eliminated the complications which occur in the transfer of phosphate through cell walls. The increase in phosphate first in endogenous phosphorylation, especially in media with KCl, can be explained by competition of two or more enzynes for the substrate, one of which catalyzes inclusion of P_i in metaphosphate, a second, inclusion in components which are hydrolyzed by TCA. According to this assumption, the first enzyme (metaphosphatase) is activated by K^+, and the second, on the contrary, is inhibited. Since the metaphosphate reaction proceeds more slowly than the reaction suppresed by K^+, it would be more correct to speak of the inhibition of endogenous phosphorylation by potassium.

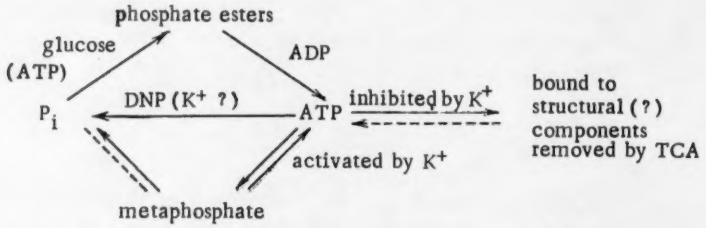

Fig. 6. Scheme of phosphate metabolism in yeast cells.

In the presence of substrate, at first glucose is phosphorylated and there is an increase in amount of acid-soluble phosphate esters or ATP, with which phosphate is transferred to metaphosphate. This possibility is confirmed by the discovery of an enzyme which reversibly transfer P from ATP to metaphosphate (Gabriel [26]). In this case, in accord with the assumption of activation of metaphosphatase by K ions, we find an increase in metaphosphate content and hence an increased level of P_i. We are dealing with an equilibrium in which an increased P_i concentration is not accompanied by an increased concentration of all the other intermediate products (Fig. 6).

This scheme explains the effects noted during aerobic metabolism. Under anaerobic conditions, however, there is almost no difference in content of metaphosphate in media with NaCl or KCl. The difference between P_i content in media with NaCl and with KCl found under these conditions can be explained by activation of some sort of other compound of the polyphosphate type.

Also, we cannot exclude a specification of K^+ on other enzymes; but for this it would be necessary to carry out a large number of kinetic measurements. In favor of the above scheme of activation of the reaction in which ATP takes part is also the fact that the total number of high-energy bonds formed in a medium with KCl is only slightly greater than in a medium with NaCl in the presence of substrate, but it is much greater in purely endogenous metabolism.

In connection with the determination of P_i content, it is possible to object, along with Lynen and Holzer [27] that removal of P_i with TCA gives especially a structural phosphate (aerobically above 50%) which is split in the extraction by TCA. Although this objection must be considered, there is no basis for believing that the amount of structural phosphate thus split would be different in media with NaCl and with KCl.

Probably K^+ acts on the energy metabolism of yeast so that during phosphorylation of endogenous substrate it permits better use of the energy of metabolism. In the phosphorylation of glucose K^+ stimulates the inclusion of P_i in metaphosphate. In this and other cases, as the kinetic data also show, the stationary level of P_i cannot serve as a measure of its effect.

From these results it follows that the effect of K^+ cannot be explained by its action on hexokinase, localized at the surface of the cell, as Rothstein [28] assumed.

SUMMARY

We have determined the inorganic phosphate, labile phosphate, total phosphate, and metaphosphate in extracts from bakers' yeast.

We have studied the effect of Na^+ and K^+ in the culture medium on the phosphorylation processes; we have calculated the rate constants of these processes under different conditions.

We have studied the effect of urethane, DNP, and arsenates as inhibitors of phosphorylation, and also the effect of cyanides. We have compared the action of K^+ and Rb^+.

Starting from these results, we can give an assumption that K^+ acts on the phosphorylation of endogenous and added substrates as a regulator of the inclusion of inorganic phosphate in the structural components and the formation of high-energy bonds.

I express deep thanks to Dr. A. A. Kleinzeller for valuable advice and guidance in this work. I am grateful for the technical help of Ia. Shevets.

Received January 31, 1958

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THE CONTENT OF SUBSTANCES WITH SULFHYDRYL GROUPS IN DIFFERENT PARTS OF THE CENTRAL NERVOUS SYSTEM

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In studies of the amount of glutathione (or other substances with SH groups) and their transformations in organs, relatively little attention has been devoted to their content and metabolism in the central nervous system (CNS), while the metabolism of glutathione in other animal organs has been much more thoroughly studied [1,2].

In the majority of studies, the authors have determined the glutathione in the brain as a whole, without consideraing the differences in its content in the gray and white matter, and also in different parts of the CNS. Thus, Binet and Weller [2] reported that in the brains of rats and guinea pigs the amount of reduced glutathione was ~ 70 mg%; Shantavy reported about the same results for rabbits [3]. McIlwain and co-workers [4] found the content of glutathione in rat brains to be 103 mg% and in guinea pigs, 80 mg%. Thus the central nervous system as a whole has about the same glutathione content as the lungs, heart muscle, or a suspension of erythrocytes. The amount of glutathione in the brains of different animals was studied by Bierich and Rosenbohm [5]. In the course of ontogenetic development the glutathione (and ascorbic acid) content in some part of the brain was determined by Rozenfel'd and Gol'dman [6]. The work of Gatsaniok [7] was also devoted to this question. Douglas and Mortensen [8] found by intracisternal or intraperitoneal injection of labeled glycine, that the transformation of glutathione occurred much more slowly in the brain than in the liver. However, the authors found that in the brain the labelled component of glutathione was only glycine, while in the liver the isotope was found also in other amino acids. Koshtoiants [9] ascribed to glutathione and substances with SH groups an important role in transmission of nervous stimulation.

As is evident from the preceding, there has still been no work in which there was a systematic study of the content of sulfhydryl substances in the separate parts of the central nervous system. Therefore we decided to determine by different methods the amount of substances with free SH groups (on the basis of reduced glutathione) in different parts of the CNS.

METHODS

For a qualitative determination of the topographic distribution of the substances with free SH groups in brain tissue we used the well-known reaction with sodium nitroprusside, which has recently been modified by Watts, Erdman, and Wentworth [10] or by Rausch and Hamer [11]. The principle of this method is the reaction of free SH groups with sodium nitroprusside in an alkaline medium. In our experiments slices of brain tissue were sprayed by an atomizer with an ammonia solution of sodium nitroprusside and we estimated the intensity of the violet color directly or by color photography.

Quantitative topographic determination was carried out by the classical method of Binet and Weller [12] in the modification of Chernokh [13] or by polarography [14].

The polarographic method was chosen because it did not require denaturation which might free bound sulfhydryl groups. Also, the polarographic method could determine some free SH groups of proteins.

The parts of the brain to be studied were ground with sand in a porcelain mortar at room temperature; the homogenate was diluted with definite amounts of modified Tyrode solution, pH ~ 6.7. After removal of oxygen by passing in nitrogen or hydrogen, the homogenate was studied in a Kalousek polarographic vessel with inserted anode and cathode.

We carried out the topographic determination of the content of sulfhydryl substances on pig brain. The animals were killed by bleeding after preliminary electric shock. The brain was removed from the cranium, cooled as fast as possible to 3°, and kept at this temperature until treatment (1-3 hours); after removal of the meningeal membrane, the brain was analyzed qualitatively and quantitatively. For qualitative analysis sections of brain tissue were prepared about 1 cm thick; for quantitative analysis separate parts of the brain were isolated in the amount of 1-2 g of fresh tissue.

For determination of dry residue, separate parts of the brain tissues were sampled with weights of about 100-300 mg, which were quickly weighed on a torsion balance and dried at 105° to constant weight.

DISCUSSION OF RESULTS

The gray matter and basal ganglion were stained by an alkaline solution of sodium nitroprusside more intensely and rapidly than the white matter in the animals studied. We could also find a difference in intensity of color in different parts of the white matter. Thus, for example, the white matter of the hemisphere was more intensely colored than the oblongata substance and the spinal cord. Of the different parts of the gray matter, the most intensely staining was the basal ganglion and the cerebellar cortex.

The amount of reduced glutathione which we could determine in the separate parts of the pig brain by the polarographic method and also by the method of Binet, Weller, and Chernokh is given in the table.

Amount of Reduced Glutathione in Different Parts of the Central Nervous System

Part of CNS studied	% dry substance	Polarographic method			Iodometric method		
		$\bar{x}$	s	$\bar{x}'$	$\bar{x}$	s	$\bar{x}'$
Cerebral cortex	14.7	54.3	±7.4	380	35.6	±10.6	242
White matter of cerebral hemisphere	31.9	44.4	±5.0	139	32.0	± 7.5	100
Nucleus caudatus (with part of the internal capsule)	24.3	67.3	±5.1	277	43.9	± 7.9	181
Cerebellar cortex	15.5	63.7	±7.3	411	30.7	± 8.9	198
White matter of cerebellum	33.7	40.5	±3.4	120	27.7	± 4.6	82
Pons varolii	28.6	43.0	±6.0	150	28.3	± 6.9	99
Medulla oblongata	27.9	40.9	±5.6	147	23.2	± 9.9	83
Spinal cord	32.9	32.4	±5.3	99	24.6	± 8.8	75

Designations: $\bar{x}$ – arithmetical average of data from 10 animals;
s – square of average deviation [15];
$\bar{x}'$ – arithmetical average $\bar{x}$ calculated on dry substance.

The values for the dry residues of different parts of the brain given in the table agree well with the literature data [16-18].

As the table shows, the determination of SH groups by the two methods gives somewhat different results. The amount found by the method of Binet and Weller is approximately 30-50% lower than that from the polarographic data. As yet we cannot explain this difference. It is evidently not connected with chance errors, but with the fact that each of these methods determined a somewhat different total of substances which contain SH groups. The polarographic method determines substances with SH groups in undenatured tissue, and so, perhaps, the results of this method correspond better to conditions in vivo. The quantitative method with sodium nitroprusside also indicates a higher content of SH groups in the gray matter of the brain, which agrees with the results of the polarographic method. In the studies by these two methods we are dealing with more or less native tissues and first of all in the presence of protein, which, however, in the method of Binet and Weller, is removed.

We found by the polarographic method a higher quantity of SH groups in all parts of the gray matter studied (nucleus caudatus, cerebral and cerebellar cortex) than in the parts of the brain in which white matter predominated. The difference between the content of SH groups of gray and white matter was statistically significant. By the method of Binet and Weller we obtained the highest values in the nucleus caudatus. By the same method we also found higher values for SH group content in the cerebral and cerebellar cortex than in the corresponding white matter, but this difference was less pronounced.

Since the separate parts of the brain differ from each other in water content, we also calculated the content of substances with SH groups for the dry substance. After this calculation, the difference between gray and white matter was still more evident. Here the first place was occupied by the cerebral cortex, the second by the cerebellar cortex, and the third by the nucleus caudatus. However, it is important to note that the data for the nucleus caudatus is distorted by the impossibility of isolating this nucleus completely. Contamination by the white matter of the internal capsule is also evident from the amount of its dry substance, which lies between the amounts characteristic of the gray and white matter (table). These three parts of the brain differ considerably from the other parts of the brain in their high content of sulfhydryl compounds. A second group consists of the white matter of the hemispheres, medulla oblongata, and pons. The lowest amount of substances with SH groups is found in the case of white matter of cerebellum and spinal cord.

Our results agree well with the data of various authors concerning other metabolically important substances or processes. Thus, for example, Richter and co-workers [19] and Fischer and co-workers [20] determined that methionine labeled with radioactive sulfur was included more rapidly in the composition of the gray matter of the brain and chiefly of the cerebellum than in the other, white matter. The consumption of oxygen per unit weight and time was also found to be greatest in the cerebellar cortex, in the nucleus caudatus, and in the cerebral cortex [16, 21].

SUMMARY

We have determined by different methods the content of substances with free SH groups in different parts of the central nervous system. The greatest amount of such substances was found in the gray matter (cerebral and cerebellar cortex and basal ganglion) and the lowest in the parts with predominantly white matter (white matter of the cerebral hemispheres, medulla oblongata, pons, white matter of the cerebellum and spinal cord). The polarographic method gives higher results than the method of Binet and Weller.

We thank the administration of the city slaughterhouse and the veterinarians for supplying us with the material needed for our work.

Received February 10, 1958

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DETERMINATION OF SULFHYDRYL COMPOUNDS IN MICRO- AND ULTRAMICRO- AMOUNTS OF TISSUE

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A number of methods have been published for the determination of SH-groups in biological material.

Up till recently, the most widely used one was that of Anson and Mirsky, in particular, in Tsyperovich and Loseva's modification [1]. Of recent years increasing use has been made of the method of amperometric titration of SH-groups, in different variants of the original method of Kolthoff and Harris [2]. Many authors consider that this method is the most accurate and the most convenient [3-6]. Savich and Iakovlev [7] have shown that amperometric titration is applicable not only to determination of SH-groups in soluble proteins, but also to their titration in tissue homogenates.

These methods are, however, only applicable to the determination of SH-groups in comparatively large amounts of material, of the order of 10-50 mg, which renders them inapplicable to quantitative histochemical researches, which require the determination of the chemical composition of microstructural tissue elements. In view of this requirement of quantitative histochemistry we undertook the elaboration of a method for the determination of sulfhydryl compounds in micro- and ultramicro-amounts of tissue. The method was based on amperometric titration of tissue homogenates with silver nitrate solutions. Its adaptation to microanalytical conditions involved the design of special apparatus and procedures, which are described in the present paper.

DESCRIPTION OF METHOD

Titration Apparatus

The basic difficulty of titration of ultramicro-amounts of sulfhydryl compounds lies in the need to use small volumes of dilute $AgNO_3$ solution, with which only a shallow inflexion is seen in the current strength curve at the equivalent point, so causing a considerable lowering of the accuracy of the results. In order to overcome this difficulty we designed a special amperometric titration vessel, with a capacity of 0.3 ml (Fig. 1). When even ultramicro amounts of sulfhydryl compounds are titrated in such vessels a relatively high concentration of Ag ions is obtained as soon as the equivalent point is passed, because of the small volume of the system, and this gives a sufficiently sharp end-point.

The titration vessel also serves as a microhomogenizer. For this purpose a conical depression is made in the bottom of the vessel, ground to fit a ground-glass piston b, fixed to a glass tube. This is connected with the spindle of a motor by a rubber sleeve. A platinum electrode a is fused into the glass tube, directly above the cone of the piston; the electrode is 25 mm long (coiled around the tube) and 0.5 mm thick. The electrode is connected through a mercury contact c with an iron wire d, which is soldered to the spindle of the motor, through which the platinum electrode is connected with the electrotitration circuit. Electrolytic connection with the reference electrode is achieved through a side arm e filled with KCl – agar gel. The opening in the side of the vessel, in contact with the agar gel, should have a diameter of not less than 0.1-0.2 mm. Should the opening be bigger than this, diffusion of Cl ions from the agar to the solution may become sufficiently great to interfere with the results of the titration.

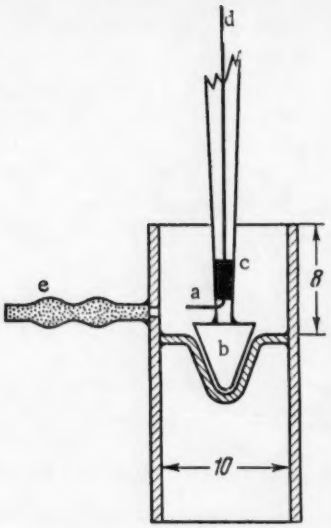

Fig. 1. Vessel for microtitration of SH-groups in tissue homogenates. a) Platinum electrode, b) homogenizer cone, c) mercury contact, d) iron wire, e) agar bridge. Dimensions shown in mm.

We used a horizontal ultramicroburet, fixed to a graduated panel, and calibrated, for microtitration of SH-groups with 10^{-3} M $AgNO_3$. We found that the most convenient volume for the buret was of the order of 30 μl, with 0.1 μl divisions of the scale. Delivery from the buret was effected either by adjusting its inclination by means of the special screw h (Fig. 2), or by manipulating the 3-way tap g, which also serves for filling the buret. During titration the tip of the buret dips into the solution under analysis.

Changes in strength of current during titration were measured by means of an M-91 microamperometer, reading up to 1 μa, with the scale graduated in 0.01 μa divisions.

The reference electrode is that usually applied to amperometric titration of SH-groups, viz., Hg – HgI_2. The platinum electrode was rotated at the rate of 750-800 rpm, and the rate could be adjusted by the aid of a rheostat inserted into the feed circuit of the collector motor.

The electrical circuits were of the type ordinarily used in such installations [2, 7].

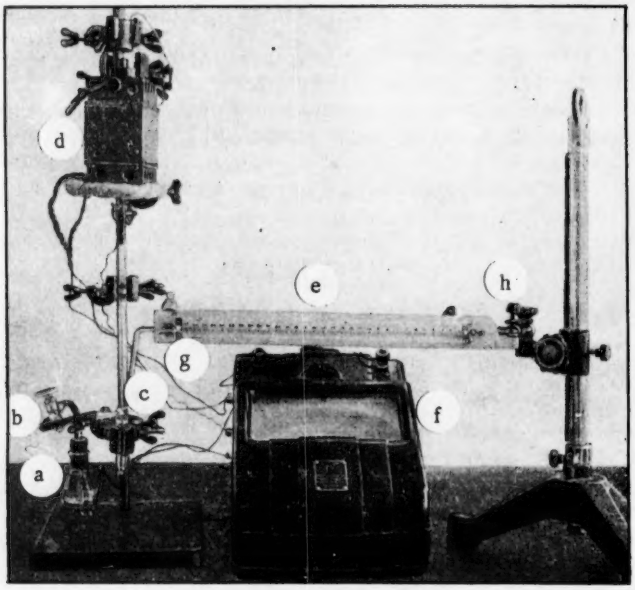

Fig. 2. Equipment for microtitration of SH-groups. a)Reference electrode, b) salt bridge, c) vessel for microtitration and homogenization, d) motor for rotating the platinum electrode, e) ultramicroburet, f) microamperometer, g) three-way buret tap, h) screw for adjusting the angle of inclination of the buret.

A photograph of the equipment is reproduced in Fig. 2.

Working Procedure, and Testing of the Accuracy of The Method

For titration of various sulfhydryl compounds (cysteine, glutathione, sodium 2,3-dimercaptopropanesulfonate – "Unithiol", blood serum, brain tissue) we used ammonia buffer, containing 0.01 M NH_4OH and 0.08 M NH_4NO_3. Samples of solutions of thiol compounds and of blood serum were taken up in automatic, calibrated self-filling pipets, and transferred quantitatively to titration vessels containing 0.3 ml of buffer solution. Slices or pieces of brain tissue were weighed on a torsion balance and transferred quantitatively to titration vessels containing 20-30 μl of buffer, and were homogenized by rotating the cone. The vessel was then slightly lowered, and the volume of buffer was made up to 0.3 ml. After closing all the circuits we recorded the constant strength of current with the motor working, lowered the tip of the buret into the solution, and started the titration. We used 10^{-3} M and $0.5 \cdot 10^{-3}$ M $AgNO_3$, standardized by Mohr's method.

The results of titration were, as usual, plotted on a graph connecting strength of current with volume of $AgNO_3$ solution added. We found that there was a certain degree of instability of current strength toward the end of the titrations; after adding a portion of $AgNO_3$ solution the current strength reached a maximum value, and then began to fall. For this reason we recommend, in order to get reproducible results, that the amperometer readings should be taken at some fixed time interval after addition of each portion of $AgNO_3$ solution.

We tested the accuracy of the method by titrating known amounts of glutathione. Fig. 3 shows the curve obtained for titration of 0.01205 μmoles of glutathione with 10^{-3} M $AgNO_3$. The mean of five parallel determinations of SH-group content was 0.01199 μmoles (i.e., 99.5%), with a mean square deviation of 0.075.

This result shows that the method is sufficiently accurate, with a relatively small scatter of individual results obtained in parallel titrations, indicating good reproducibility.

The results obtained for the SH-group content of cysteine, Unithiol, and blood serum, using the ordinary macro-method and our ultramicro-method, are given in Table 1.

TABLE 1

Results of Amperometric Titration of SH-groups in Solutions of Thiol Compounds and in Blood Serum, Using the Macro- and the Micro-method.

Method of titration	Cysteine		Unithiol		Cat blood serum	
	amount titrated	SH-groups found, μmoles/ml	amount titrated	SH-groups found, μmoles/ml	amount titrated	SH-groups found μmoles/ml
Macro-method	1 ml	0.650	2 ml	0.447	0.5 ml	0.750
Micro-method	24.1 μl	0.625	24.1 μl	0.432	11.8 μl	0.732
% Deviation		−3.8		−3.4		−2.4

As is shown by the data of Table 1, the results given by the two methods are in sufficiently good agreement.

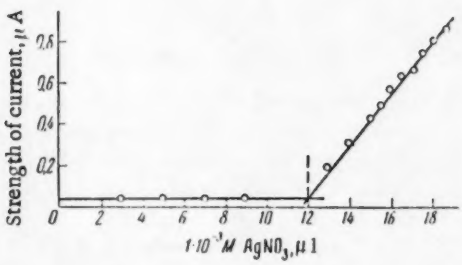

Fig. 3. Curve for titration of 0.01205 μmoles of glutathione.

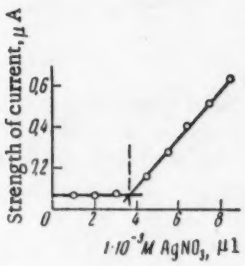

Fig. 4. Curve for titration of 6.9 μl of cat blood serum.

The end point obtained in the titration of micro- and ultramicro-amounts of biological material is quite sharp. This is illustrated by the curve of Fig. 4, representing titration of 6.9 μl of cat blood serum with 10^{-3} M $AgNO_3$. We found that even smaller amounts of serum (1-2 μl) may be titrated, using $5 \cdot 10^{-4}$ M $AgNO_3$. Here, too, the end point is sufficiently sharp.

Table 2

SH-Group Content of Different Levels of the Motor Cortex of a Cat (SH-group content, in μmoles per 100 mg of tissue)

Superficial layer	Deep layer
0.86	1.05
0.92	1.00
0.89	0.95
0.80	0.04*
Mean: 0.87	1.01

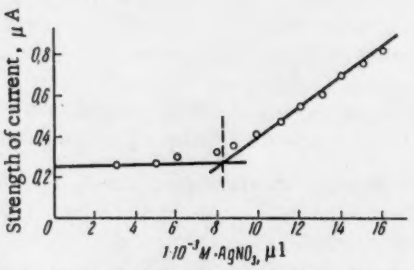

Fig. 5. Curve for titration of a slice of cat's cerebral cortex. Weight of slice 0.8 mg, thickness 50 μ.

As was implied above, our object in working out the ultramicro-method was to enable us to determine the SH-group content of a single histological section of tissue.

Figure 5 shows a typical amperometric titration curve, for titration of a homogenate of a section (thickness 50 μ, weight 0.8 mg) of cerebral cortex of a cat. It appears from the graph that the end point obtained is as definite as for titration of soluble thiol compounds. It is also clear that the amount of tissue could be still further reduced by a factor of 3-4, using equipment of the sensitivity described. It follows that one can, if necessary, perform the analysis on sections 12-15 μ thick, containing $1.5\text{-}2 \cdot 10^{-3}$ μmoles of SH-groups. It is therefore possible, in conjunction with the results of histological examination of the tissues, to assess the histochemical nature of their microstructural elements.

As an illustration of the application of our method of quantitative histochemical analysis, we present the results of SH-group determination in different layers of the motor zone of the cerebral cortex of a cat. A cylindrical block of tissue was taken, and sections 50 μ thick were cut parallel to the surface of the cortex, by means of a rotary freezing microtome, at −10°; the sections were weighed, transferred quantitatively to the titration vessel, and their sulfhydryl group content was determined as described above.

Table 2 presents the results of analysis of sections taken from the superficial (molecular) layer of the cortex, and from a deeper layer containing a high proportion of neurons.

Our results show that the deeper layers of the cortex, which contain the bulk of the nerve cells, have a higher SH-group content than do the more superficial layers, which contain terminal ramifications of long dendrites of the pyramidal cells, glial syncytium, and tangential fibers. These differences in SH-group content are not large, but they are significant.

We intend, in our future researches, to make a systematic examination, layer by layer, of the different zones of the cerebral cortex, as well as of other parts of the brain.

SUMMARY

An ultramicro-amperometric titration method is described, used for determination of amounts of the order of 1.5-2 μmoles of SH-groups present in tissue extracts, fluids, and homogenates. The method permits the analysis of single histological sections of tissues, giving results which can be applied to the histochemical evaluation of the results of microscopic examination of stained tissue sections.

Received February 15, 1958

* As in original.

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REVERSIBLE PHOTOREDUCTION OF CHLOROPHYLL, AND SENSITIZED REACTIONS IN SUGAR BEET LEAF HOMOGENATES

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The mechanism of the photosensitizing action of chlorophyll on processes of hydrogen (electron) transfer involving "storage" of light energy in reaction products has been established experimentally in our laboratory, and it has been shown that the photosensitizing action of chlorophyll depends basically on its reversible photoreduction [1]. These experiments were conducted with solutions of chlorophyll or its analogs in organic solvents. It remained to elucidate whether chlorophyll could take part in such transformations when combined, as it is in its natural state, with proteins and lipids.

When investigating the state of chlorophyll in leaf homogenates [2, 3] we observed that in a number of plant species part of the chlorophyll was present in a photochemically unstable state; this form of chlorophyll is rapidly bleached during illumination, and its properties resemble those of chlorophyll dissolved in organic solvents. The amount of this form of chlorophyll is particularly high in homogenates of the leaves of sugar beet, goosefoot, poplar, etc. [3]. We were able to observe the reversible photochemical transformation of chlorophyll in homogenates of the leaves of these plants [2].

The participation of chlorophyll in photochemical hydrogen transfer processes taking place in solutions is clearly manifested in a reducing medium, in the presence of ascorbic acid; it was hence desirable to attempt to provide such reducing conditions in leaf homogenates. With this object, we ground the leaves up with sodium ascorbate, and centrifuged the product. Electron microscope examination of the resulting homogenate, which we call "green solution", revealed the presence of chloroplast fragments and of whole and broken-down grana. As hydrogen (electron) acceptors we took the same compounds as were used by us previously for solutions, viz., reversibly reducible safranin T ($E_0' = -0.3$ V), riboflavin ($E_0' = -0.22$ V), and the irreversibly reducible azo dye, methyl red.

The properties of chlorophyll contained in the green solutions were compared with those of chlorophyll in colloidal solutions.

EXPERIMENTAL MATERIALS AND METHODS

Our experiments were performed chiefly on sugar beet leaves ("Verkhniachka" variety), grown in the open in September and October, or in greenhouses under fluorescent light during the winter.

The green solution was in most cases prepared in the following way: the central vein was removed from the leaves, and 1.5 g portions of leaf were ground up, with cooling, with 10 ml of M/15 Na_2HPO_4 containing 4 g of sodium ascorbate. The resulting brei was strained through canvas, and the juice was centrifuged at 3500 g for 10 min. The supernatant fluid was poured off, and was diluted with glycerol, in the proportion of 4:6. This solution was further diluted with an aqueous glycerolic solution of sodium ascorbate (100 ml of solution contained 60 ml of glycerol, 40 ml of M/15 Na_2HPO_4, and 4 g of sodium ascorbate), until an extinction coefficient of K = 0.6 - 0.7 (light path 1 - 1.2 cm) was obtained at the wave length of maximum absorption by chlorophyll at the red end of the spectrum. The solutions were stored in the refrigerator.

Green solutions without ascorbate were prepared as described previously [2, 3].

Colloidal solutions of chlorophyll a + b were prepared by adding 3 parts of water to 1 part of a concentrated solution of the pigment in acetone. We added 0.03-0.1 ml of concentrated colloidal solution to 10 ml of aqueous glycerolic sodium ascorbate solution, until the value of K was 0.5-0.7 at the red absorption maximum of chlorophyll. The aqueous glycerolic solution contained 60 ml of glycerol, 40 ml of M/15 Na_2HPO_4, and 1.6 g of sodium ascorbate per 100 ml. Acetone present in the colloidal solution was removed by evaporation under reduced pressure.

The experimental conditions, including exposure to light, and the spectrophotometric measurements, were the same as in our previous papers [2, 3]. In addition, we exposed the solutions to red light (RG-2 filter), of an intensity of 10^6 erg/cm^2/sec.

Sodium ascorbate was prepared in the following way. A solution of ascorbic acid in distilled water was neutralized with solid sodium bicarbonate (1 mole of $NaHCO_3$ per mole of ascorbic acid), and the salt was precipitated by adding excess of ethanol, and leaving overnight in the refrigerator. The solution was then filtered, and the precipitate was washed with ethanol and dried in vacuum. The aqueous sodium ascorbate solution had a pH of about 7.

REVERSIBLE PHOTOREDUCTION OF CHLOROPHYLL

We have shown that illumination of aqueous glycerolic green solutions in vacuo leads, when these solutions have a high content of the photochemically labile form of chlorophyll, to a reversible lowering of the red absorption maximum of chlorophyll, due to reversible reduction of a small part of the pigment [2]. This effect can be observed repeatedly with exposures of short duration. Addition of neutral ascorbate solutions (3.5×10^{-3} M) to the green solutions renders the process more fully reversible. We were able, in our earlier experiments, to show that illumination of the solutions led to a fall of up to 3% in absorption at the red maximum.

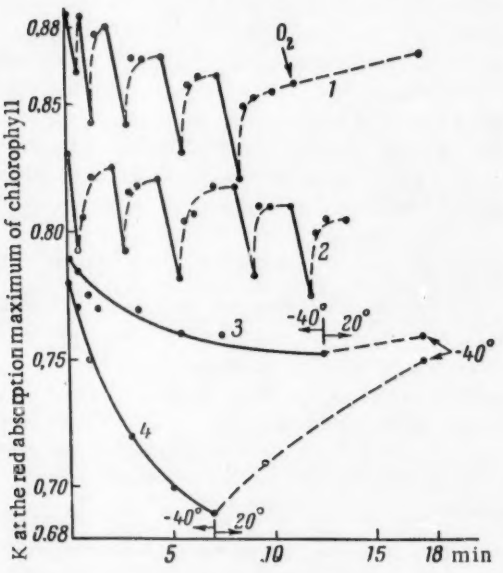

Fig. 1. Reversible photoreduction of chlorophyll in green solutions prepared from sugar beet leaves, and containing sodium ascorbate. 1) With exclusion of air (676 mμ), 2) in presence of air (676 mμ), 3) without air, at −40° (676 mμ), 4) with 5% pyridine at −40° (673 mμ). In this and subsequent figures the continuous lines refer to periods of illumination, and the broken lines to the dark interval; the time of admission of air is indicated by arrows. Figures in parentheses refer to the position of the red absorption maximum of chlorophyll.

The use of green solutions containing a large excess of sodium ascorbate enabled us to observe the phenomenon of reversible reduction of chlorophyll in such "solutions" in a more pronounced form. The differential spectrum, which shows the differences in the spectra before and after illumination, shows a maximum at 670 mμ, from which it follows that the bleaching effect is due to the "monomeric" form (Fig. 10). Illumination of such "solutions" in the absence of air leads to a considerable lowering of K at the red absorption maximum of chlorophyll, which is partly reversed in the dark and after admission of air (Fig. 1). The high ascorbate concentrations present in these experiments inhibited oxidation reactions in the green solutions, showing that the reversible change in K observed in the presence of air is also due to reversible reduction (Fig. 1). The same effects were seen when ascorbate was added to aqueous glycerolic green solution immediately before the experiment.

In order to compare the properties of chlorophyll in its natural state and after isolation from the plant, we exposed a colloidal solution of chlorophyll a + b, containing sodium ascorbate, to light, in the absence of air. This also resulted in a slight (by

1-2%) partly reversible lowering of K, at the absorption maximum of chlorophyll.

Effects of Various Factors on the Reversible Transformation of Chlorophyll

Temperature. The reversible changes in the K of chlorophyll at its absorption maximum are inhibited at low temperatures. A slight lowering of K at the red absorption maximum of chlorophyll was noted when green solution containing sodium ascorbate was illuminated at −40°. When the "solution" was allowed to stand at room temperature, and its optical density was then measured at −40°, partial regeneration of chlorophyll from its intermediate reduction products was observed. When green solution containing 5% pyridine was illuminated at −40°, we observed considerable lowering of K at the absorption maximum of chlorophyll, with considerable regeneration at room temperature (Fig. 1). The increased effect at low temperatures is probably due to inhibition of back reactions. Concentration of sodium ascorbate. We examined the effect of varying the concentration of sodium ascorbate added to the green solution (prepared without ascorbate) on the reversible transformations of chlorophyll taking place during illumination. We found that $7 \cdot 10^{-3}$ M ascorbate caused a perceptible increase in reversible photoreduction, as compared with the original solution. As ascorbate concentration was increased to 0.35 M we found a progressive increase in the irreversible lowering of K at the red absorption maximum of chlorophyll. The amount of regenerated product rose with increasing time of standing in the dark. It may be supposed that the high concentration of chlorophyll favors the fuller reduction of chlorophyll, required for the reverse transition to the initial state of a series of dark reactions. The maximum effect is achieved at an ascorbate concentration of 0.07 M (50 mg of ascorbate in 4 ml of solution).

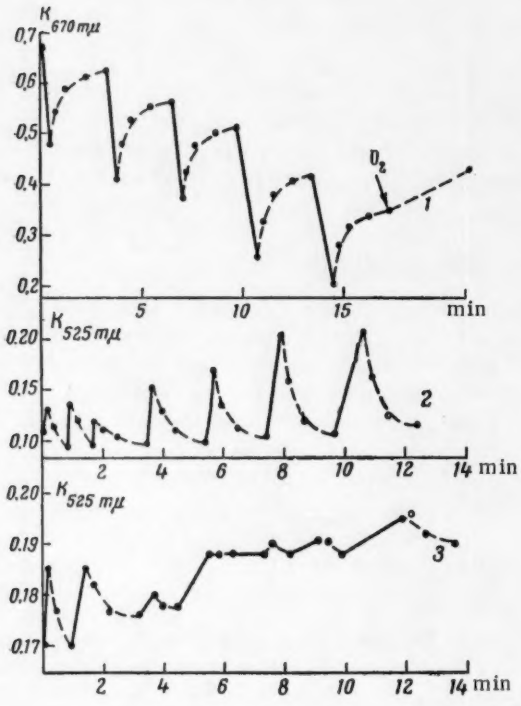

Fig. 2. Photoreduction of chlorophyll in green solution prepared from sugar beet leaves, and containing sodium ascorbate and pyridine, in vacuum. 1 and 2) 50%; 3) 20% pyridine.

Addition of pyridine. As was shown in an earlier research, pyridine exerts a powerful disaggregating effect on chlorophyll in its natural state, even at low concentrations [4]. It was for this reason of interest to investigate the effect of different concentrations of pyridine on the reversible photoreduction of chlorophyll in green solution containing sodium ascorbate.

Lynch and French [5] examined the effect of adding small amounts of pyridine on the photochemical properties of chloroplast suspensions containing ascorbic acid, and found that illumination of such solutions did not cause any changes in the spectrum at 672 and 525 mμ, with addition of up to 50% pyridine to the suspensions.

We added pyridine, in concentrations of 1, 5, 20, and 50%, to green solution prepared from sugar beet leaves, with addition of sodium ascorbate, and measured the changes in K at the red absorption maximum of chlorophyll, when the "solution" was illuminated in vacuo. The absorption maximum of such suspensions was at about 677 mμ. No perceptible effect was given by 1% pyridine on the change in K at the absorption maximum of chlorophyll during illumination, or on the position of the absorption maximum (about 676 mμ). The maximum was shifted to 675 mμ in 5% pyridine, which also caused a perceptible increase in photochemical reduction. The absorption maximum of chlorophyll was at 671 mμ in 20% pyridine. Reversible lowering of K was much greater in such solutions. In 50% pyridine the binding between protein and chlorophyll is loosened,

and a solution of chlorophyll in aqueous pyridine is formed, with maximum absorption at 669 mμ. Illumination of such solutions in vacuo leads to a considerable lowering of absorption in the red part of the spectrum, as also at 525 mμ, i.e., photoreduction takes place with production of the red reduced form (Fig. 2). The process is rapidly reversed in the dark, probably because of the high water content of the systems, which causes acceleration of the reverse reaction.

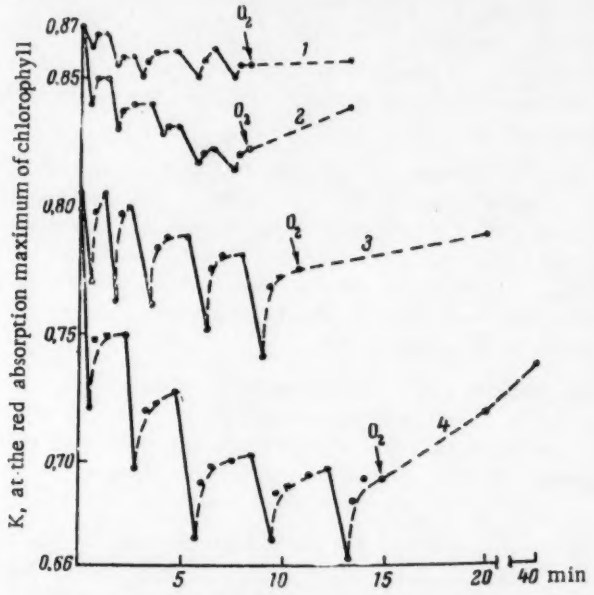

Fig. 3. Photoreduction of chlorophyll in green solution prepared at different seasons of the year from sugar beet leaves, and containing sodium ascorbate. 1) Dec. 16, 1957 (677 mμ), 2) the same, in presence of 5% pyridine (673 mμ), 3) Sept. 30, 1957 (676 mμ), 4) the same, in presence of 5% pyridine (673 mμ).

Physiological conditions. As we have shown in an earlier paper [2], the physiological conditions under which the plant is grown affect the spectral and photochemical properties of chlorophyll in homogenates. This was also found in the present research. Green solutions prepared from sugar beet plants grown in the open in September and October contained chlorophyll in a more active state than did such solutions prepared from plants grown in a greenhouse in December (Fig. 3). Addition of small amounts of pyridine to green solution prepared from leaves grown in October caused more extensive breakdown of chlorophyll complexes than was the case with solutions prepared from leaves grown in December, as described in the preceding paragraph. Here we found that the red absorption maximum of chlorophyll was shifted to 673 mμ in 5% pyridine, and to 671 mμ in 10% pyridine. Fig. 3 shows the difference between the photochemical properties of chlorophyll in green solutions prepared from leaves grown in October and December, and containing 5% pyridine.

Plant species. We have shown that homogenates prepared from the leaves of different plant species differ in their content of photochemically labile chlorophyll [3]. In this research we investigated the capacity of chlorophyll in green solutions prepared from different species of plants to undergo reversible photochemical transformation. Wheat and beans were taken for the experiments, as being plants with a low content of the labile form [3].

We found that reversible reduction of chlorophyll by ascorbate took place in green solutions prepared from wheat and bean leaves, although to a smaller extent than with sugar beet leaves. Addition of pyridine did not enhance the effect, even at concentrations of 20%, which shifted the absorption maximum to 671 mμ. In 50% pyridine the natural state of the chlorophyll is changed, and in such solutions illumination causes ordinary photoreduction, characteristic of real solutions of the pigment.

SENSITIZED REDUCTION OF DYES

A detailed study has been made in our laboratory of the photosensitizing effect of chlorophyll on the reduction of certain dyes (safranin and methyl red), and of riboflavin and diphosphopyridine nucleotide [1]. In these experiments, we found a rapid fall in the extinction coefficient at the absorption maximum of the substrate when its solution, containing also chlorophyll and ascorbic acid, was illuminated with red light in vacuo; absorption at the chlorophyll absorption maximum did not change until all the substrate (electron acceptor) had undergone reduction.

Our green solutions, which contained photochemically active chlorophyll which could be reversibly reduced when exposed to light, provided suitable material for the study of the effect of chlorophyll in its natural state on the photosensitized reduction of dyes.

The green solutions used in these experiments were prepared with sodium ascorbate, by the method described above. Solutions of safranin T, riboflavin, and methyl red were made up in M/15 Na_2HPO_4, in such concentrations that when we added 0.02-0.04 ml of the solution to 4 ml of green solution the extinction coefficient at the given wave length fell within the range 0.6-1.0. Reduction of safranin T was measured from the lowering of its absorption maximum at 530 $m\mu$. With methyl red the measurements were made near the isobestic point, at 460 $m\mu$ [6]. The measurements with riboflavin were also made at 460 $m\mu$, since absorption by chlorophyll is very high at its absorption maximum of 445 $m\mu$.

Reversible reduction of safranin T, and irreversible reduction of methyl red, were found in our experiments. Reduction of riboflavin could be seen only in solutions containing 5% pyridine at a temperature of from $-10°$ to $-40°$; this effect should be ascribed to inhibition of back-reactions. At room temperature we were able to observe only inhibition by riboflavin of reversible lowering of K at the absorption maximum of chlorophyll (Fig. 4).

Reduction of the substrates was also found in the presence of air, and this effect can be ascribed to suppression of oxidative processes by the high concentrations of ascorbic acid present. We found in control experiments that reduction of these substrates did not take place in green solutions not containing sodium ascorbate, or in aqueous glycerolic solutions without chlorophyll, when exposed to red light.

When chlorophyll-sensitized reduction of the substrates by ascorbic acid in solutions containing pyridine took place, we were unable, during the initial stages of the light reaction, to observe any change in absorption by chlorophyll at its absorption maximum, although the process involves the stage of photoreduction of chlorophyll [1]. This is because the reduced form of chlorophyll reacts rapidly with the electron acceptor molecules of substrate, in a reversible way. When the sensitized reduction reaction was conducted in green solutions of a high sodium ascorbate content the changes at the absorption maximum of chlorophyll were not entirely suppressed. With methyl red, they were lowered by about a half (Fig. 4). This diminution was found to occur both in the presence and the absence of air. Addition of 50% pyridine to the green solutions caused no further suppression of the changes in chlorophyll at its absorption maximum, although it is evident that in this case we were dealing with an aqueous glycerolic solution of chlorophyll. Similar effects were seen with safranin T and riboflavin, in systems containing 5% pyridine to (Fig. 4).

Safranin. Illumination in vacuo of green solution prepared from sugar beet leaves, and containing sodium ascorbate and safranin T, caused lowering of K at the absorption maximum of the dye (530 $m\mu$). The reverse process took place in the dark (Fig. 5). Under these conditions the reaction of reduction of safranin was fully reversible. The dark back reaction of oxidation of leuco-safranin was usually completed within 1.5-2 min. It was accelerated by addition of 5% pyridine. Reduction of safranin could also be observed in the presence of air, although the magnitude of the effect was in this case smaller.

No measurable changes were seen at 530 $m\mu$ when the experiments were performed in the presence of atmospheric oxygen at $-40°$. Progressive, slow reduction of safranin was observed when the reaction was conducted at 40° in vacuo, and the reaction was reversed when the temperature was raised (Fig. 5).

We next examined reduction of safranin in aqueous glycerolic green solutions containing sodium ascorbate after preliminary heating in a water bath for 30 min at 70° and 90° (this treatment does not lead to formation of floccules of denatured protein). In such systems we found only partly reversible reduction of the dye, which

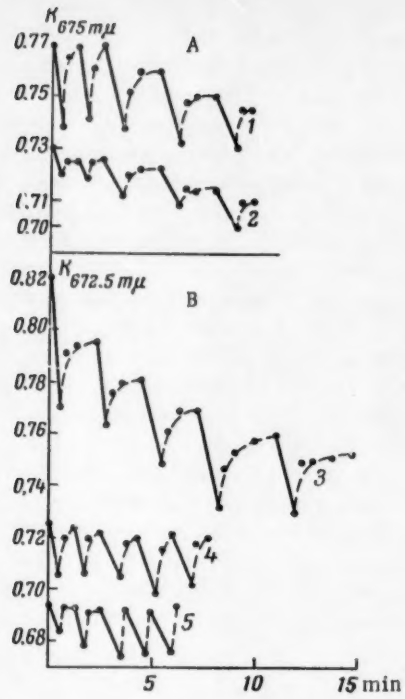

Fig. 4. Changes in extinction at the red absorption maximum of chlorophyll during illumination of green solutions containing sodium ascorbate in vacuo and in the presence of hydrogen acceptors. A: 1) green solution, without additives 2) with methyl red $K_{460m\mu} = 3$); B: 3) green solution containing 5% pyridine, 4) the same with safranin ($K_{530m\mu} = 3.5$), 5) the same, with riboflavin ($K_{445m\mu} = 2$).

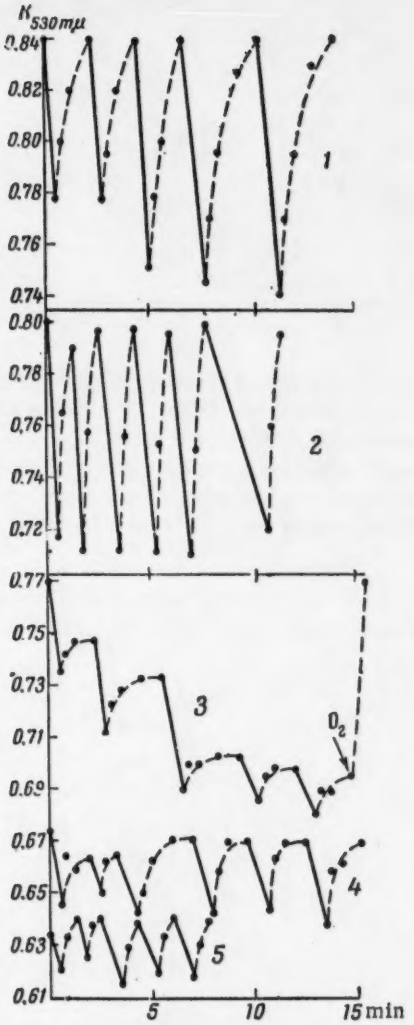

Fig. 5. Photosensitized reduction of safranin (with sodium ascorbate, in vacuo). 1) Green solution prepared from sugar beet leaves, 2) green solution prepared from sugar beet leaves and containing 5% of pyridine, 3) green solution from sugar beet leaves, previously heated to 70°, 4) colloidal solution of chlorophyll a + b, 5) green solution prepared from bean leaves. The systems were illuminated with red light.

went further, however, than in unheated solution (Fig. 5). The reverse reaction was accelerated in the presence of oxygen, and when the system was shaken with air the extinction coefficient at the absorption maximum of the dye rose to its initial value.

Reduction of safranin was also found in green solution prepared from bean leaves, as well as in aqueous glycerolic solutions of chlorophyll a + b containing sodium ascorbate. It is evident from Fig. 5 that in these cases the amount of dye reduced is much smaller than in the experiments with green solutions from sugar beet leaves.

Methyl red underwent reduction in green solutions prepared from sugar beet leaves, to which sodium ascorbate had been added before the addition of the dye (Fig. 6). Reduction of the bulk of the dye usually took place within 2-3 min , in both presence or absence of air. The rate of reduction was greatly diminished at −40° (Fig. 7).

We found that the rate of reduction of methyl red in aqueous glycerolic green solutions was directly proportional to their ascorbate concentration (Fig. 8).

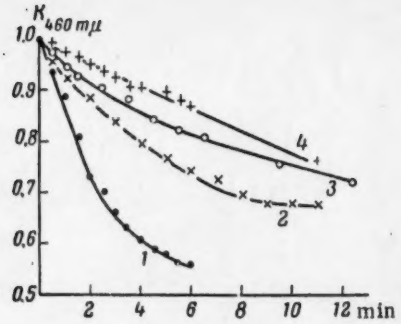

Fig. 6. Photosensitized reduction of methyl red (in vacuo and with sodium ascorbate). 1) Green solution prepared from sugar beet leaves, 2) colloidal solution of chlorophyll a + b, 3) green solution from wheat leaves, 4) green solution from sugar beet leaves, exposed to red light before addition of sodium ascorbate.

We had found that addition of pyridine in small amounts, insufficient to bring chlorophyll into solution, greatly intensified reversible photochemical reduction of chlorophyll in green solutions prepared from sugar beet leaves, containing sodium ascorbate. It might have been expected that addition of pyridine to such solutions would also accelerate reduction of methyl red. We found, however, that addition to green solution prepared from sugar beet leaves of from 1 to 50% pyridine did not greatly affect the rate of reduction of methyl red (Fig. 9). Exposure of aqueous glycerolic green solution not containing sodium ascorbate to red light of high intensity (10^6 ergs/cm^2/sec) for 10 min., in presence of air, caused decomposition of about 15% of the chlorophyll, judging from the lowering of K at the red absorption maximum. The resulting solution had largely lost its ability to sensitize reduction of methyl red, as compared with the initial unilluminated solution (Fig. 6). This phenomenon resembles the suppression of photochemical activity of green solutions, as expressed by the phenol-indophenol reaction after preliminary exposure to red light of high intensity in presence of air; this effect was

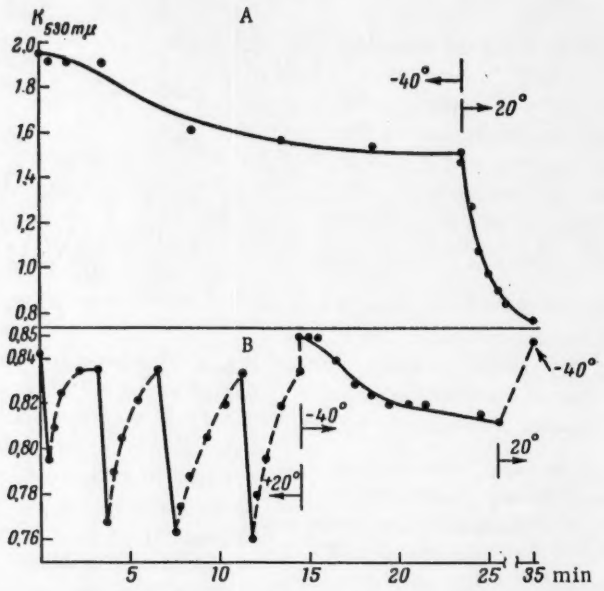

Fig. 7. Photosensitized reduction of dyes in green solutions prepared from sugar beet leaves, with addition of sodium ascorbate, at low temperatures. A) Methyl red, B) safranin.

briefly described by us in an earlier paper [7]. We have ascribed suppression of photochemical activity to decomposition of the labile monomeric form of the pigment during illumination; this form is probably essential for the photochemical stage of the process of oxygen production. The same interpretation may be applied to the present experiments, in which reduction of methyl red did not take place after preliminary illumination of the green solution.

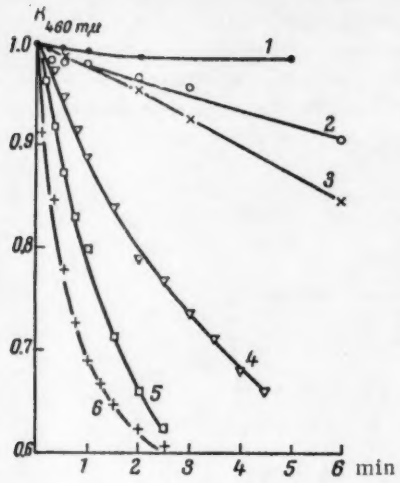

Fig. 8. Photosensitized reduction of methyl red in green solutions prepared from sugar beet leaves, at various concentrations of sodium ascorbate. 1) Without ascorbate, 2) 7×10^{-3} M, 3) 1.4×10^{-2} M, 4) 7×10^{-2} M, 5) $1.4. \times 10^{-1}$ M, 6) 3.5×10^{-1} M ascorbate.

Reduction of methyl red can also be observed in green solutions containing sodium ascorbate, prepared from bean and wheat leaves, although the velocity of reduction of the dye was very low (Fig. 6). Addition of pyridine to the green solution from bean leaves led to acceleration of this process. In the limiting case, with 50% pyridine in the solution, the velocity of reduction of methyl red approached that found with sugar beet leaf green solution (Fig. 9).

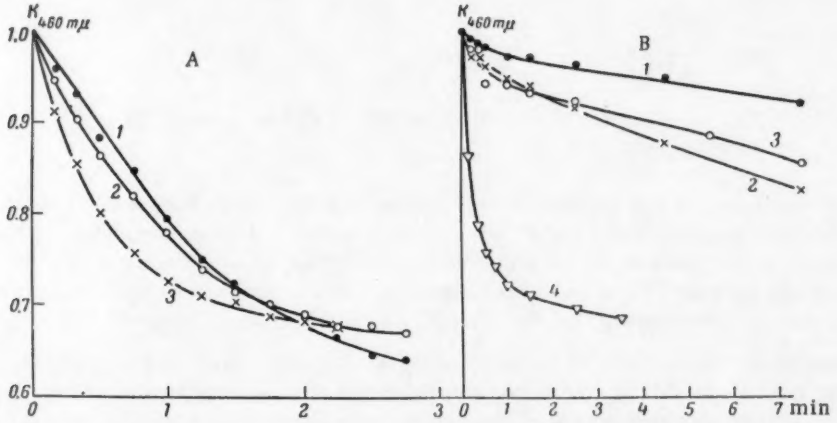

Fig. 9. Effect of pyridine on photoreduction of methyl red in green solutions containing sodium ascorbate, in vacuo. A – Green solution prepared from sugar beet leaves: 1) without pyridine, 2) 1% pyridine, 3) 50% pyridine. B – Green solution prepared from bean leaves: 1) without pyridine, 2) 5% pyridine, 3) 20% pyridine. 4) 50% pyridine.

Reduction of methyl red also took place in colloidal solutions of chlorophyll a + b, containing sodium ascorbate. In this case, the velocity of reduction of the dye was comparable with that found in green solutions prepared from bean and wheat leaves.

Changes in the Extinction Coefficient in the Green Part of the Spectrum

Numerous papers have been published in recent years, dealing with reversible spectral changes in the green part of the spectrum, taking place during illumination of green leaves or suspensions of algae [8-12].

It has been shown by a number of workers that absorption in the 510-540 mμ band rises as a result of illumination. Their experiments demonstrated the existence of a correlation between increase in absorption in this region of the spectrum and the extent of lowering of the red absorption maximum of chlorophyll [11], and also with diminution in fluorescence [12]. The substances responsible for the changes in absorption in the green region of the spectrum have, however, not yet been identified. It is possible that more than one substance is involved [10, 11].

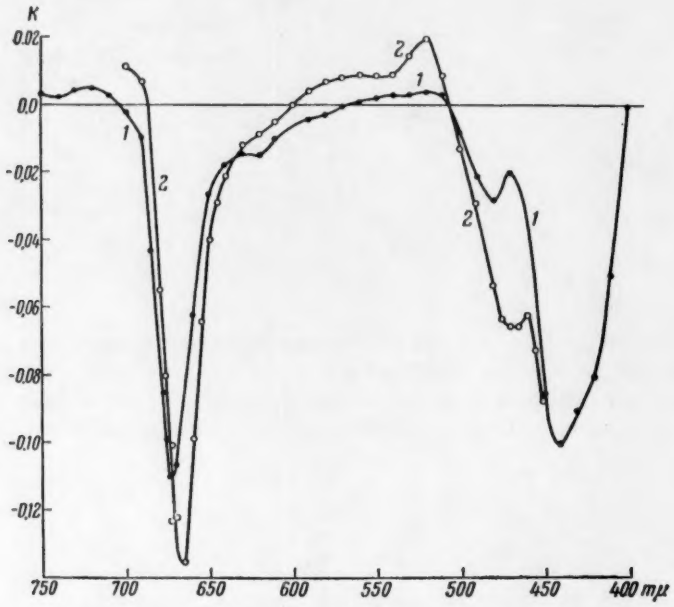

Fig. 10. Differential absorption spectra. 1) Without pyridine, 2) with 5% pyridine.

Our experiments did not show any considerable changes in absorption in the green part of the spectrum when we illuminated green solutions prepared from sugar beet leaves, and containing sodium ascorbate, with exclusion of air. It should, however, be noted that with the Beckmann spectrophotometer only changes in the extinction coefficient exceeding 1% of the initial value before illumination can be taken as significant, i.e., only effects ten times greater than the Duysens effect [8] are capable of being perceived.

We found a certain increase in K in the 510-540 mμ band, but this effect only slightly exceeded the error of measurement, although the fall in K at the red absorption maximum reached a value of 15%. We were thus able to observe reversible photoreduction of chlorophyll, not associated with any marked changes in the green region of the spectrum.

A slight rise in absorption at 525 mμ was seen when the green solution contained 5% pyridine (Fig. 10). Higher values of K were also found with solutions containing 20% pyridine, at 525 mμ; this effect was reversible during the initial period of illumination (Fig. 2). The differential spectrum, being the difference between the spectra before and after illumination of the solutions in absence of air, shows two maxima, one at 540 mμ, and the other in the 515-520 mμ band. These did not disappear when air was admitted, showing that the spectral changes were irreversible. Green solutions containing 50% pyridine gave a sharp maximum at 525 mμ after illumination, which disappeared almost completely about a minute after switching off the light (Fig. 2). This acceleration of the reaction rate, as compared with chlorophyll solutions, is probably ascribable to the high water content of the green solutions.

DISCUSSION OF RESULTS

Our experiments demonstrated reversible changes in optical density at the red absorption maximum of chloorphyll, amounting to 5-6%, when green solutions prepared from sugar beet leaves, and containing excess

of sodium ascorbate, were illuminated. The reverse reaction could be perceived for a time of 1-1.5 min , which distinguishes the effect observed by us from the numerous measurements of differential spectra in living leaves and in algal suspensions [8-12].

Coleman, Holt and Rabinowich [9], and also Streler and Lynch [11] have reported reversible changes in the optical density of suspensions of Chlorella, in the red part of the spectrum. Witt found a fall in fluorescence of chlorophyll in Chlorella suspensions, with simultaneous increase in absorption at 530 $m\mu$ [12], reported in Duysens' papers [8].

In these researches, the changes in optical density at 510-530 $m\mu$ exceeded the fall in optical density at the red absorption maximum of chlorophyll. It is for this reason unlikely that the chlorophyll which was transformed in these experiments was converted into the red reduced form. Judging from our experiments on chlorophyll solutions, the ratio K_{670}/K_{530} should have had a value of 2-3. It has been shown in our laboratory that, under appropriate conditions, photoreduced forms are produced, not giving an absorption maximum at 525 $m\mu$, and, moreover, that sensitization in solutions involves the primary formation of the reduced form of chlorophyll [13], not giving an absorption maximum at 525 $m\mu$. It is more probable that the rise in absorption in the green region of the spectrum, as shown in the differential spectra, is due not only to transformation products of chlorophyll, but also to transformations of other, as yet unknown, hydrogen acceptors, such as members of the cytochrome series. The reversible transformations of chlorophyll in the red region of the spectrum should, however, be ascribed, as in our experiments, to reversible photoreduction. All these problems require further investigation.

The results of our experiments on the progressive disaggregation of chlorophyll in leaf homogenates by pyridine should be examined in the light of these concepts. We found in these experiments that the magnitude of the changes in absorption by chlorophyll at its absorption maximum varied parallel with the pyridine concentration of the solutions. We did not, however, find any marked increase in absorption at 525 $m\mu$ following illumination of the systems, at any concentration of pyridine up to passage of all the chlorophyll into solution (Fig. 2), showing that under these conditions photoreduction was not associated with formation of the red reduced form of chlorophyll.

The reversible transformation of chlorophyll observed by us in green solutions should be considered as involving the disaggregated monomeric form of chlorophyll, the amount of which in the homogenates varies for different plant species, and with their physiological state, as has been shown in our earlier papers [2, 3]. The nature of the monomeric form present in homogenates requires further study; in particular, it is necessary to investigate the possibility that it may have been produced during the homogenization process.

Photosensitizing activity of chlorophyll was found in all the preparations studied by us, the highest activity being observed in sugar beet leaf homogenates, which have a high content of the monomeric form of chlorophyll. It appears, however, that both the monomeric and the aggregated forms of chlorophyll take part in the reaction, as was shown by the experiments with colloidal solutions. All our experiments gave quantitative reduction of safranin during illumination, with complete reversal of the reaction in the dark. We were not able directly to establish reduction of riboflavin, possibly because of the high velocity of the reverse reaction. At room temperature, and using green solutions containing 5% pyridine, all we could find was a fall in K at the red absorption maximum of chlorophyll, due probably to reaction of the reduced form of chlorophyll with riboflavin. At low temperatures we found considerable lowering of K in the riboflavin absorption band.

In contrast to the Hill reaction, the sensitizing effect observed in our experiments was not dependent on chlorophyll being in its natural condition, or on the integrity of the biocatalytic system of the material, since the sensitizing effect is not abolished by heating the systems or by the presence of small amounts of pyridine in them, whereas the Hill reaction is completely inhibited by such treatment.

SUMMARY

Illumination of sugar beet leaf homogenates containing sodium ascorbate causes reversible changes in the optical density of the systems at the red absorption maximum of chlorophyll. This effect is due to reversible photochemical reduction of chlorophyll.

Photosensitized reduction of safranin T, methyl red, and riboflavin takes place in sugar beet, bean, and

wheat leaf homogenates, and in colloidal solutions of chlorophyll a + b, in the presence of sodium ascorbate; this process probably involves intermediary photoreduction of chlorophyll.

These effects vary greatly, according to the plant species and to the physiological state of the plant from which the homogenates are prepared. These differences are related to the content of active monomeric form of chlorophyll in the homogenates.

Received February 21, 1958

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•• See English translation.

PHYSICOCHEMICAL PROPERTIES OF ACYLASE

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From the time of detection of acylase in swine kidney by Price and Greenstein in 1948 [1], there have been a number of studies devoted to this subject [2-11]. However, these investigations were conducted on the unpurified preparations and accordingly, up to now, data are lacking concerning a number of physicochemical properties of this enzyme. Using an acylase preparation of high activity obtained by a previously described method [12], we were successful by further purification in obtaining the enzyme in satisfactorily pure form. The present paper comprises a study of a number of physicochemical properties of acylase.

EXPERIMENTAL

Method of Acylase Isolation

The final purification of the acylase preparation obtained by the method we developed [12] was conducted by electrophoresis on starch. For this purpose we employed the apparatus of usual type with a starch block of 25 x 13 x 2 cm. A reversible electrode (silver chloride, silver) in a saturated solution of sodium chloride was used. A repeatedly washed starch was suspended in 0.05 M phosphate buffer at pH 7.1. After shaping the starch block the semi-liquid mixture of acylase and starch was placed in it [13] (about 300 mg of acylase preparation, preliminarily dialyzed against the buffer). Electrophoresis was conducted for 21-22 hours at 4°, using a 65 ma current. After completion of the test, the starch block was cut into portions. Each portion was extracted 2-3 times with water. In the water eluates obtained, the protein content, acylase, and cathepsin activity were determined spectrophotometrically. Figure 1 shows the quantitative distribution of protein in relation to the distance from the point of application of the original preparation in the direction of the positive electrode. The acylase activity was found to be concentrated in the fractions 15-23 cm from the point of application, and the cathepsin activity at 3-7 cm. The water fractions containing the acylase (18 to 23 cm) were concentrated in a cellophane bag with an air current at room temperature, dialyzed against distilled water and then freeze-dried under vacuum. By this procedure there was obtained 100 mg of pure acylase; by comparison with the original material its activity increased by 20-30% (the activity was verified by action on acetyl-alanine and was 33,000 μmol/mg nitrogen per hour).

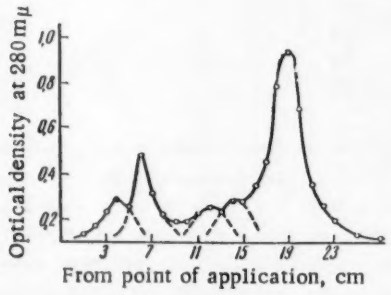

Fig. 1. Protein distribution of original acylase preparation following electrophoresis on starch block.

Electrophoretic Studies

Electrophoretic investigation of acylase in an 0.1 M phosphate buffer, pH 7.1, was conducted in a Tiselius apparatus at 1.5°. As can be seen from the electrophoregrams in Figure 2, the acylase preparation obtained by

electrophoresis on starch (Fig. 2 b) yields a symmetrical peak which attests to the essential homogeneity of this protein. By comparison is shown the electrophoregram of the original preparation obtained after fractionation by acetone (Fig. 2 a).

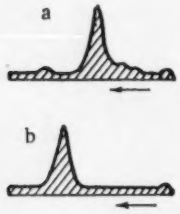

Fig. 2. Electrophoresis diagram (ascending boundary) original and purified acylase preparations. a) Original preparation after 240-min electrophoresis; b) purified preparation after 360-min electrophoresis.

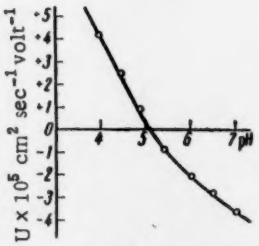

Fig. 3. Electrophoretic mobility of acylase in relation to pH.

TABLE 1

Magnitude of Electrophoretic Mobility of Acylase Preparations at Various pH Values

pH	0.1 M buffer	Gradient potential V/cm	Mobility 10^{-5} cm^2 V^{-1}	
			ascending	descending
3.93	Acetate	5.69	4.36	4.16
4.44	"	5.56	2.77	2.48
4.90	"	5.58	0.99	0.92
5.44	"	5.61	−0.95	−0.94
6.00	Phosphate	5.70	−2.02	−2.11
6.50	"	5.68	−2.74	−2.80
7.00	"	5.71	−3.57	−3.71

To determine the isoelectric point of acylase, solutions of the enzyme were prepared in buffer mixtures having an ionic strength of 0.1 and various pH [14]. The protein concentration in the solutions was 0.4% in all cases. Prior to the test the acylase solution was dialyzed against buffer. The electrophoretic mobility was calculated from the data as to the descending boundary, since in this case more precise data are obtained than in using the ascending boundary of the electrophoregram [15]. The magnitude of the electrophoretic mobility at various pH's is given in Table 1 and Figure 3. From these data it can be seen that the isoelectric point of acylase is at pH 5.1. It should be noted that at pH 4.9 the solubility of acylase decreased and a portion of the protein precipitated out.

Studies on Acylase Sedimentation and Diffusion

Acylase sedimentation was investigated on the Svedberg ultracentrifuge [16] at a rotor speed of 56,000 rpm at a temperature of 19.5-20°. A solution in 0.1 M phosphate buffer, pH 7.1, was used. The method of combined scales [17] was used as a control on the sedimentation, and also an optical system with oblique slits and cylindrical lenses. The coefficient of sedimentation was calculated graphically, using the formula:

$$S = \ln (x_2/x_1) / (t_2 - t_1)\, \omega^2,$$

where x_2 and x_1 equal the distance from the center of the precipitation boundary and the two axes of rotation at times t_2 and t_1, and ω is the angular velocity. The coefficient of sedimentation was brought to standard conditions (water at 20°) and extrapolated to infinite dilution (Fig. 4). The sedimentation constant thus obtained was $S_{20} = 5.6$ Svedberg units. It should be noted that the sedimentation diagram obtained 100 minutes and more after achieving fixed speed indicates the presence of a small quantity of substances sedimenting at a lower speed (Fig. 5).

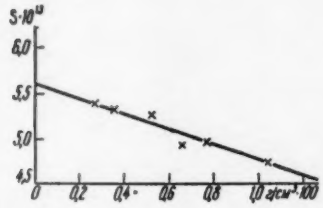

Fig. 4. Graph of sedimentation constant of acylase in relation to concentration.

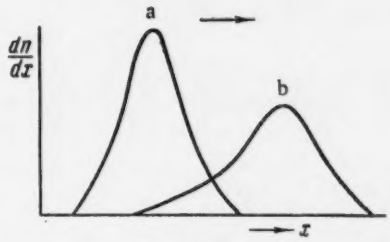

Fig. 5. Sedimentation diagram of acylase: a) After 40 min; b) after 100 min (at rotor speed of 56,000 rpm, t – 20°)

Diffusion studies were carried out in a Lamm apparatus [17] at a temperature of 20 ± 0.003°. The same acylase solutions were employed as in the sedimentation studies. The diffusion coefficient was calculated by the formula $D_A = (A/N)^2/4\pi t$ from the graph of the relationship $(A/N)^2$ to time t (A = area, N – maximum coordinate of diffusion curve, t – time). The coefficient of diffusion was adjusted to standard conditions (water at 20°). No essential relationship of diffusion to protein concentration in the solution was found (Table 2). The diffusion constant we obtained was $D_A = 4.41 \cdot 10^{-7}$ cm²/sec. The specific volume of acylase was determined by pycnometer and calculated according to the formula

$$V = \frac{1}{m_0}\left[v - \frac{1}{g}(m - m_0)\right],$$

where m_0 is the weight of solvent, m – weight of solution, g – protein concentration in g/ml, v – pycnometer volume. For our acylase preparation there was found V = 0.74.

TABLE 2

Diffusion Constant of Acylase Preparation in Relation to Concentration

Concentration in g/100 ml	$D_A \cdot 10^7$
1.2	4.43
0.9	4.44
0.7	4.37
Average:	4.41

The sedimentation and diffusion constants obtained, and also the magnitude of the specific volume, made it possible to calculate the molecular weight of acylase. The calculated molecular weight (M) was carried out by the Svedberg formula $M = RTs/D(1 - v)$. M was found to be 119,000.

In addition, the relationship of the friction coefficient $f/f_0 = 1.48$, was calculated and from this the semi-axis relationship b/a = 9 (on the assumption that the acylase molecule is hydrodynamically equivalent to an elongated ellipsoid).

Viscosity Studies

The viscosity was measured at 20° with an Ostwald viscosimeter (bulb volume 0.8 cm³, capillary radius 0.15 mm) at various gradient flow rates. The magnitude of the specific viscosity at various acylase concentrations was not dependent on the gradient flow rate, which indicates the absence of structural viscosity and the considerable intermolecular interaction. From the graph of the relationship between the reduced viscosity (η_{ud}/c) and the concentration there was found the magnitude of the characteristic acylase viscosity $[\eta] = 0.11$ (the concentration expressed in g/100 cm³ – Fig. 6). By Sim's $\nu = \frac{[\eta] \times 100}{v}$ (where ν is the coefficient of viscosity, and v is the specific protein volume) the value ν was found to be 14.9. From tables [18] of the

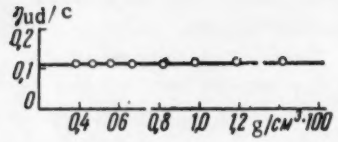

Fig. 6. Relationship of characteristic viscosity of acylase preparation to concentration.

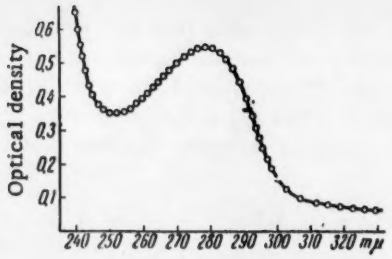

Fig. 7. Acylase absorption spectrum.

relationship of the axial ratio to the coefficient of viscosity we found the magnitude b/a = 10.6, which is close to that obtained from the data of sedimentation and diffusion.

Absorption Spectrum

The acylase absorption spectrum was determined with an SF-4 spectrophotometer. As can be seen from the absorption curve shown in Fig. 7, the maximum acylase absorption is found in the region of 280 mμ. Thus, acylase has an absorption spectrum characteristic of other simple protein substances.

DISCUSSION

Although the acylase preparation we isolated was homogeneous in electrophoresis studies, an ultracentrifuge investigation indicates the presence of some impurities with a lower sedimentation rate. It is difficult to come to any final conclusion as to the nature of these impurites. However, it must be taken into account that preliminary acylase purification was conducted under conditions which might bring about partial denaturation of the protein (heating for 5 min at 60°, precipitation with 40-50% acetone), and it is therefore possible that the inhomogeneity during sedimentation depended upon some changes in the enzyme molecule. These circumstances make it impossible to consider the value for molecular weight as highly accurate. Accordingly, it would be more proper to speak of an order of magnitude.

SUMMARY

An acylase I preparation has been isolated by starch electrophoresis. Its activity with acetyl-alanine as substrate is 10 times as high as that of a similar acylase I preparation previously obtained by Greenstein. The preparation thus obtained proved electrophoretically homogeneous with an isoelectric point at pH 5.1.

The sedimentation constant S_{20} = 5.6 Svedberg units, the diffusion constant D = $4.41 \cdot 10^{-7}$ cm^2/sec and the specific volume V = 0.74. The molecular weight of acylase I computed from these data was about 119,000, and the axis ratio b/a = 9.

The viscosity and absorption spectrum of the acylase preparation were also studied.

We express thanks to V. O. Shpikiter for his assistance in these studies.

Received March 1, 1958

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THE PREPARATION OF HIGHLY PURIFIED DIPHOSPHOPYRIDINE NUCLEOTIDE BY MEANS OF A CHROMATOGRAPHIC COLUMN WITH THE ANIONITE EDE-10-P

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Methods for isolating and purifying diphosphopyridine nucleotide (DPN)have been described by many authors [1-10].

It has been shown [1, 4] that the most suitable material for isolation of DPN is yeast.

It has often been observed that the yield and purity of the DPN preparation depend on the type, conditions of growth, and freshness of the yeast. In most of the methods the first stage of purification is the extraction of DPN from the yeast cells by hot water. Extraction with a mixture of alcohol and ether [8] has not been much used. As a following step, fractionation using salts of heavy metals has often been used for purification [1, 2, 4, 5]. This widely used method for obtaining a highly purified coenzyme, DPN, results in great difficulty and small yields. However, the accumulation of a "primary" preparation which contains 10-20% of the dinucleotide is often carried out by the method of Williamson and Green [2],who isolated the silver salt of DPN after they had removed the phosphate esters, proteins and other inert substances from the yeast extract by precipitation with basic lead acetate.

The ability of certain types of activated charcoal (Nuchar C, Nuchar C-190, Norite S x 30 special etc.) to adsorb DPN reversibly has been observed and has led to the use of this property for purifying DPN [3, 5, 6, 9]. The removal of the cozymase from the charcoal is carried out by repeated extraction with an aqueous solution of pyridine with the addition of amyl alcohol; when the nucleotide adsorbed on the charcoal is removed, there is considerable loss of DPN. Many types of carbon are unsuitable for the removal of the coenzyme from yeast extracts. There has also been little use of the method based on the principle of counter-current separation which was suggested [7] for obtaining small amounts of a highly purified preparation of DPN.

At the present time, apparently the most effective and suitable method for the purification of DPN is that based on the principle of ion exchange column chromatography. In 1951, Neilands and Akeson [9] increased fourfold the purity of a preparation which contained 20% DPN before purification, by chromatography on a column of the anionite Dowex-1 in acetate form. The same procedure was the basis of the method of Wallenfels and Christian [11] which permitted them to obtain a preparation of DPN with a very high degree of purity (up to 100%). In another method [2] for obtaining highly purified DPN, chromatography on a column of Dowex-1 in formate form was used.

We set up the problem of using a domestic ion exchange resin for obtaining a highly purified preparation of DPN. Below we give a method for obtaining a 90-100 percent preparation of DPN by chromatography on the anionite EDE-10-P in the acetate form.

EXPERIMENTAL PART

Starting material. For the extraction of DPN we mostly used freshly pressed bakers' yeast (Derbenevskii Yeast Works). In some experiments, DPN was isolated from unpressed bakers' yeast from the same factory or from brewers' yeast (Badaev Works) which was also a fully satisfactory material.

The yeast extract was obtained by extraction with hot water [5,9]. Five kg of previously ground pressed bakers' yeast was suspended in 100-150 g portions in 5 liters of distilled water with strong stirring and heated to 90°. When all the yeast was suspended in water (this procedure could be carried out in an enamel bucket by passing through the suspension a strong current of steam in order to prevent any marked fall in temperature of the suspension of yeast in water), the mixture was kept for 5 min at 90°. Then the yeast suspension was cooled quickly to 30-40° with a mixture of snow or ice and salt (usually the cooling took 15-20 min), and centrifuged at 3000 rpm, for 15 min. The precipitate was discarded. The content of DPN in the yeast extract was determined by the fluorometric method in the modification of Carpenter and Kodicek [13].

This "primary" preparation of DPN (unpurified cozymase) was treated by different methods.

1. Adsorption of DPN on charcoal [3, 9]. ST charcoal used in the sugar industry was activated according to Robson and Selim [14]. To the yeast extract obtained as described above, a clear yellow-brown liquid with pH 6.8-7.0, was added charcoal, calculated on the basis of 50 g of freshly activated charcoal to 5 liters of extract. The mixture was stirred mechanically for 30-40 min at room temperature, and then the charcoal was removed by filtration or centrifuging and washed with 2-3 volumes of water. The DPN was removed from the charcoal by three successive extractions with strong mechanical stirring by 1.5, 1, and 0.5 liters of 15% aqueous pyridine solution in the course of 1.5, 1, and 0.5 hours, respectively. The resulting eluate was evaporated in a vacuum to 1/10 to 1/12 the original volume. The DPN was removed, after acidification of the cooled solution by concentrated nitric acid to pH 2.5, by precipitation with four volumes of cooled anhydrous acetone [9]. The resulting preparation contained 7-9% DPN. The yield of dinucleotide was 40-50% calculated on the total amount of DPN in the initial yeast extract. The reason for the loss was chiefly incomplete extraction of DPN from the charcoal residue. Attempts to decrease the loss of DPN (by increasing the duration and number of extractions or adding 10% amyl alcohol to the aqueous pyridine solution) did not give positive results.

2. Precipitation of DPN in the form of the silver salt [2]. To the yeast extract obtained with hot water was added 1/10 its volume of a 25% solution of basic lead acetate and after careful stirring, the precipitate was separated by centrifuging at 3000 rpm, for 20 min. The cooled supernatant liquid was acidified with 10% acetic acid to pH 6; 1/100 its volume of freshly prepared 25% aqueous solution of silver nitrate was added with strong stirring, and the mixture stood over night in a refrigerator at 3-4°. Then the precipitate was separated by centrifuging and washed with water. The silver salt of DPN was transformed into free dinucleotide by stirring a thick water suspension of the precipitated silver salt with excess cationite KU-2 in the hydrogen form (250 mesh). The cationite was separated by centrifuging, washed three times with water, and the washings combined with the supernatant liquid which had been separated from the cationite. The DPN was precipitated by adding four volumes of acetone or 10 volumes of absolute ethyl alcohol in an acid medium (pH 2-2.5). The cationite KU-2 was regenerated by repeated washing with a 10% solution of nitric acid. The preparation obtained by this process contained 4-5% DPN. The yield of coenzyme calculated on the total amount in the yeast extract was 45-50%.

3. Chromatography of DPN on anionite after passing the yeast extract through cationite [11]. The yeast extract was passed through a column with cationite SBS in hydrogen form (50-75 mesh): internal diameter of the column, 63 mm; height of resin layer, 200 mm. The extract became an opalescent, grayish white color. The pH shifted to the acid side; the content of DPN became $^1/_2$ the amount present in the initial yeast extract. The portion of coenzyme absorbed on the cationite could not be removed by acid. The loss of DPN from binding on the cationite could apparently be decreased by using a more perfect type of domestic cationite (KU-2 and others).

From the solution which had been passed through the cationite (after bringing to pH 6 with 10% sodium hydroxide) we succeeded in isolating DPN almost without loss by chromatography on a column of the anionite EDE-10-P in acetate form; the resulting preparation contained 40-45% of coenzyme I.

Purification of the "primary" preparation of DPN obtained by any of the above methods was carried out by ion exchange chromatography on a column of anionite EDE-10-P in acetate form (250 mesh). The column, whose construction and general operation were described in our previous work [15], had an internal diameter of 22-24 mm; the height of the resin layer was 250-270 mm. Transformation of the anionite from the chloride to the acetate form was carried out in the column by washing the layer with distilled water and then passing through it a 0.1 M water solution of ammonium acetate brought to pH 5.8-5.9 with glacial acetic acid. The buffer solution was passed until no more chloride ions were evolved in the solution coming from the column and the solution remained at pH 5.8-5.9; under our conditions this took 24-30 hours. After washing the column with 2-3 volumes of distilled water to remove free acetate, we considered the column ready for use.

The "primary" preparations of cozymase were dissolved in 0.1 M aqueous ammonium acetate (pH 6.8) in such a way that 100 ml of solution contained not more than 25 g of "primary" preparation. The solution was filtered through a small column (for example 10 × 10 mm) of cellulose powder [16] for the removal of insoluble contaminants. The resulting filtrate was passed through a column of the anionite EDE-10-P prepared as described above and previously washed with 1-1.5 volumes of 0.1 M ammonium acetate solution.

The optimum charge for a column of this size varied with the degree of purity of the preparation, and usually consisted of 4-10 g of "primary" preparation. After the DPN solution had been passed through the resin layer, the column was washed with 2-3 volumes of water. The solution which flowed out of the column was collected with an automatic fraction collector (of the Swedish firm LKB) set for specific times. Usually we collected fractions with volumes of 15-18 ml. We determined the DPN content of the fractions by the fluorometric method [13]. When we were satisfied that all the DPN was adsorbed on the anionite, we began the extraction of the dinucleotide with an 0.1 M acetate-ammonia buffer with pH 5.8-5.9.

As is shown in Fig. 1, which shows one of the least "sharp" peaks which we obtained in eluting DPN from the anionite EDE-10-P, full removal of the dinucleotide is attained by passing a relatively small volume of buffer through the column. As the percent content of DPN in the preparation increases during purification by ion exchange, the amount of buffer solution which must be passed through the column to remove all the adsorbed dinucleotide is increased. In our results, an increased concentration of buffer solution which produced removal was not desirable, since the excess ammonium acetate hindered still more the removal of a very pure DPN preparation.

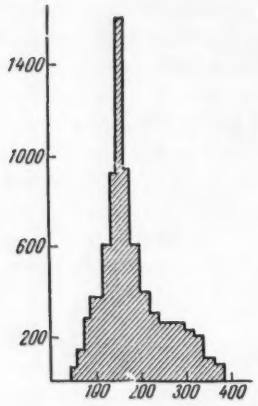

Fig. 1. Curves of yield obtained in removal of DPN from a column with anionite EDE-10-P in the acetate form (250 mesh, 24 × 265 mm). Solvent – 0.1 M acetate–ammonia buffer, pH 5.9; charge – 130 mg DPN in a 4% preparation. Found after elution 103% of the initial DPN. Abscissa – ml of buffer passed through the column. Ordinate – DPN in μg/ml, according to fluorometric determination.

It was necessary to carry out a considerable number of fluorometric determinations for orientation during the experiments; this increased the work of the chromatographic method of purifying DPN. A great aid in this respect was the use of an automatic apparatus which recorded changes in optical density in the ultraviolet part of the spectrum (230-260 mμ) in the solution which flowed from the column. Such an apparatus was constructed and completed in the Institute of Biological and Medical Chemistry of the Academy of Medical Sciences, USSR, by engineers I. V. Alekseev and B. V. Ottesen [17]. We should emphasize, however, that under the conditions described above, as Fig. 2 shows, other substances besides DPN are removed which also absorb ultraviolet light. In work with more contaminated solutions than those purified in the experiment shown in Fig. 2, the amount of these substances rose sharply. Therefore the determination of optical density in the ultraviolet of the fractions which were obtained by elution could be of value only in approximate, orienting experiments, but would not replace the quantitative determination of DPN by other methods.

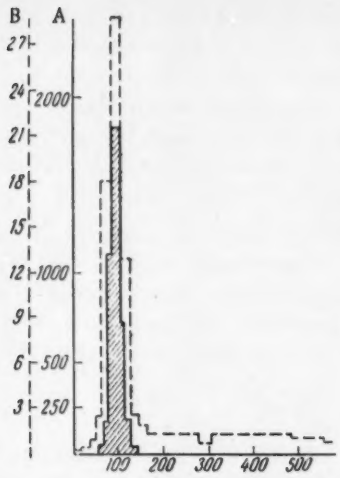

Fig. 2. Curve of yield obtained in removing DPN from a column with anionite EDE-10-P in acetate form (250 mesh, 22 × 250 mm). Concentration of pyridine nucleotide (in μg/ml) by fluorometric determination; optical density at 260 mμ of the fraction of solution emerging from the column. Solvent – 0.5 M acetate–ammonia buffer; pH 5.8; charge 75 mg DPN in a 45% preparation. Found after elution, 99.5% of the initial DPN. Abscissa – ml of buffer passed through the column. Ordinate – A – DPN in μg/ml by fluorometric determination; B – optical density at wave length 260 mμ.

After we had completed the elution, the fractions which contained more than 100 μg DPN per 1 ml were combined, dried in a vacuum at 40-45° to 1/40-1/50 of the volume of the mixture which was obtained, and the DPN was isolated by adding 10 volumes of absolute alcohol to the cooled solution with later acidification of the cooled mixture by concentrated hydrochloric acid to pH 2-2.5 (a clear blue color of Congo red paper). The acidified solution stood over night in the refrigerator at 3-4°, and the precipitate was separated by centrifuging, washing three times with 3-4 volumes of cooled 96% alcohol and dried in a vacuum desiccator over freshly ignited calcium chloride for 18-20 hours.

If necessary, the isolated preparation could be submitted to repeated chromatography on an anionite column to obtain any degree of purity, independent of the quality (strain, freshness, growth conditions etc.) of the starting yeast. These factors have an effect on the yield of DPN per kg of yeast, but not on the purity of the preparations which are obtained. In order to separate preparations of coenzyme which contain 100% DPN by the data of fluorometric investigations, we must usually chromatograph twice on the anionite. The preparations of DPN obtained after the first chromatographic purification on EDE-10-P usually contain 60-70% of the coenzyme.

Test of the Purity of the Isolated Preparations

For determination of the content of DPN in preparations purified by the method of ion exchange chromatography, we used, besides the fluorometric method, the specific enzymatic reduction of DPN with later spectrophotometric determination of the concentration of reduced dinucleotide.

As the enzyme we used alcohol dehydrase from bakers' yeast, purified by the method of Wallenfels and Sund [18]. Reduction was carried out in a semicarbazide-pyrophosphate buffer, pH 8.6 [18]. The optical density of the solutions which contained reduced DPN was determined by an SF-4 spectrophotometer in a cuvette with a 10 mm thick layer and a wave length of 366 mμ. The molar extinction coefficient at this wave length was taken equal to $3.4 \cdot 10^6$ cm^2/mole [19].

In our purified DPN preparations which contained 100% pyridine nucleotide by the fluorometric method, the enzymatic method showed 83% DPN.

DISCUSSION OF RESULTS

According to the literature data [20] the present DPN preparations contain 84% coenzyme which is active in the enzymatic test. 10-14% corresponds to the optical isomer of DPN which has no activity in the enzyme system, but is determined fluorometrically. Thus, our content of 83% enzymatically active DPN in the purified preparation of this coenzyme agrees fully with the data which have been presented.

In the purification of the "primary" preparations of DPN by ion exchange chromatography we always found quantitative removal of the dinucleotide adsorbed on the anionite EDE-10-P in the acetate form. When DPN was adsorbed directly from the yeast extract onto the anionite, its later removal was not complete; about

50% of the DPN which was contained in the initial yeast extract was irreversibly bound to the resin and the upper layer of anionite in the column was unusable. If we assume that the presence of colloidal impurities in the yeast extract could give this effect, we could repeat the experiment with vacuum drying of the yeast extract dialyzate which was obtained by rapid dialysis in a cellophane bag [21]. However, when we tried this, we found a considerable loss of DPN as a result of irreversible binding to the resin. Thus, ion exchange chromatography on EDE-10-P resin in the acetate form can be recommended only for the purification of "primary" preparation of DPN obtained by some other method, and which contains more than 2% DPN.

From a comparison of our data on the chromatography of DPN on columns of the anionite EDE-10-P in the acetate form with the data obtained in work with Dowex-1 in the acetate form [9] we conclude that under the conditions described above, this domestic anionite is suitable for replacing the anionite Dowex-1, although the chemical structure of Dowex-1 (a monofunctional strongly basic anionite of the copolymer type) is very different from that of EDE-10-P (a polyfunctional weakly basic anionite of the condensation type) [22].

SUMMARY

We have described a method of obtaining a highly purified DPN preparation from the unpurified "primary" preparation of the coenzyme by ion exchange chromatography on a column of the domestic anionite EDE-10-P in the acetate form.

I express thanks to Prof. A. E. Braunshtein for guidance in this work.

Received March 4, 1958

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A STUDY OF DEPOLYMERIZATION PROCESSES OF NUCLEIC ACIDS IN THE CELLS OF NORMAL AND CANCEROUS TISSUES

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The ability of cancer cells to grow and divide intensively is one of their most characteristic properties. At the basis of this there is apparently some peculiar metabolism of substances in cancer cells which has not been sufficiently studied and which permits them to synthesize nucleic acids and proteins even under the most unfavorable conditions.

When we study in vitro the processes of synthesis of DNA* and RNA from their common precursors, ribonucleotides, we see that in the nuclei of cancer cells DNA synthesis is possible at very low concentrations of ribonucleotides in the medium, on the order of one molecule of substrate for one nucleotide residue of nuclear DNA [1].

An entirely different relationship occurs in the nuclei of cells of highly differentiated normal tissue, the liver or the mucosa of the small intestine, where DNA synthesis is possible only with a great excess of ribonucleotides in the medium, 10-30 times more than the DNA concentration. However, the spleen, an organ with high mitotic activity, resembles tumors in its ability to use ribonucleotides for the synthesis of DNA [2].

The existing data indicate that apparently cancer cells have some sort of metabolic preference in carrying out DNA synthesis over resting cells of normal tissues.

We have assumed two possible explanations of this preference as a working hypothesis.

1. Cancer cells use, in addition to ribonucleotides, some other more complex substrate (possibly products of incomplete depolymerization of DNA) for the synthesis of DNA.

2. All the free ribonucleotides in cancer cells first undergo reduction of their ribose residues and are used for the synthesis of DNA, while in the cells of normal, highly differentiated tissues (liver, mucosa) the fate of the ribonucleotides is much more complex and varied and only a small part of them is used for DNA synthesis.

The present work is a first step in the experimental testing of these assumptions.

Privat de Garilhe and Laskowski [3] showed that two DNA depolymerases, DNA-ase I, isolated by Kunitz [4] from the pancreas, and DNA-ase II discovered by Maver and Greco [5] in the thymus and spleen of calves, differ not only in optimum pH but also in specificity to the substrate. While the "acid" DNA-ase II splits the DNA molecule chiefly into rather large fragments, oligonucleotides, the "neutral" DNA-ase I uses these large pieces of DNA as the substrate and splits them into tri-, di-, and mononucleotides.

* Abbreviations: DNA – desoxyribonucleic acid; Na DNA – sodium salt of DNA; RNA – ribonucleic acid; Na RNA – sodium salt of RNA; DNA-ase – desoxyribonuclease; RNA-ase – ribonuclease.

In our work we have tried to establish the relation of the two successive steps in DNA depolymerization in the cells of normal and cancerous tissue by determining the activity of both DNA depolymerases in the tissues which we studied and their intracellular localization.

OBJECTS AND METHODS OF STUDY

As objects of study we used transplantable tumors of rats and mice (sarcoma 45, sarcoma M-1, Ehrlich ascitic tumor) and human tumors after their operative removal (angiosarcoma, sarcoma of the soft tissues, chorioepithelioma, teratoma, seminoma). For the normal tissues we used liver, mucous membrane of the small intestine, spleen, and thymus of the rat.

The fresh tissue, previously ground up in the cold, was weighed and treated with 5 times its volume of buffered (pH 7.4) isotonic solution of sucrose with 0.018 M $CaCl_2$, and homogenized 5-16 min in a glass homogenizer, the time depending on the nature of the tissue. The homogenate was filtered, diluted with 0.25 M sucrose to a final concentration of 10% and, after separation of a small portion for determination of protein and enzyme activity, was separated into four fractions by the method of differential centrifuging [6]: nuclei, mitochondria, microsomes, and hyaloplasm. In some experiments the microsomes were not separated from the hyaloplasm and the whole centrifugate was studied. In the homogenate and its fractions we determined the protein content and the enzymatic activity of DNA-ase I, DNA-ase II, and RNA-ase. The proteins was determined by the intensity of the biuret reaction in the spectrophotometric micromethod of Goa [7], which permits determination of protein at a concentration interval of 100 to 2000 μg per ml of solution. Measurement of optical density was carried out on an SF-4 spectrophotometer at 330 mμ. For each tissue we first established a standard calibration curve.

The activity of the nucleic acid depolymerases was determined by the spectrophotometric method of Schneider and Hogeboom [8], at first on the freshly prepared fractions. Then, when we were convinced that freezing the homogenates and cell fractions at -50° did not lower the activity of the enzymes which we studied, we used material which had been thawed after deep freezing. The compostion of the incubation mixtures was:

DNA-ase I: Na DNA 1.5 mg/ml 0.2 ml; $MgSO_4$ 0.05 M – 0.2 ml; veronal buffer 0.05 M, pH 7.0 – 0.6 ml.

DNA-ase II: Na DNA 1.5 mg/ml 0.2 ml; Mg SO_4 0.05 M 0.2 ml; succinate buffer 0.1 M, pH 5.0 – 0.6 ml.

RNA-ase: Na RNA 0.2% – 0.2 ml; $MgSO_4$ 0.05 M – 0.2 ml; veronal buffer 0.05 M, pH 7.0 – 0.6 ml.

In a series of tests, these mixtures were added in the cold to 0.05-0.2 ml of homogenate or its fractions (calculated at 500-700μg protein per sample). Each pair of experimental samples had a corresponding pair of controls. In the control samples the same solutions were added to 0.3 ml of 2 M $HClO_4$. The experimental samples were incubated for 30 min at 37° in a water thermostat with stirring. The reaction was stopped by adding 0.3 ml of 2 M $HClO_4$. The samples were carefully cooled and the protein removed by centrifuging. The protein-free centrifugate was treated with water to a definite volume and measured in the spectrophotometer against the controls at 260 mμ. From the standard calibration curves established for DNA and RNA we determined the amount of products of splitting of the corresponding nucleic acids which appeared in the protein-free filtrate during incubation, and we calculated the enzyme activity in mg of nucleic acid split in 1 hour per 1 g of wet tissue. We also calculated the relative activity of the enzymes in each fraction, using the total activity of all the fractions as 100%.

We should remark that in the spectrophotometric method the determination of the activity of DNA-ase II can take account of only 60 % of the low molecular weight products of DNA-splitting which remain in the protein-free filtrate. The other 40% of large oligonucleotides is precipitated by $HClO_4$ along with the proteins and DNA and was not considered by us.

The statistical treatment of the results was carried out by the formula for small samples.

RESULTS OF THE INVESTIGATION

The normal tissues which we chose for study are distinguished by the degree of differentiation and by the mitotic activity of their cells. The most highly differentiated are the liver cells. Their mitotic activity is slight.

The cells of the mucous membrane of the small intestine have a double character: on the one hand, they have a number of special secretory functions, on the other they have the ability for active division. The spleen and thymus cells are little differentiated, but divide actively.

TABLE 1

Content and Intracellular Distribution of Protein in Normal Tissue (in mg per 1 g wet weight of tissue)

Fraction	Liver	Mucosa	Spleen	Thymus
Nucleus	17.8±1.3	32.1± 0.1	58 ± 3.6	56.8
Mitochondria	54.0±1.3	16.7± 1.1	36 ± 4.5	19.6
Microsomes)		12.4± 1.6	11.8± 2.7	
Hyaloplasma)	59.6±0.5	50.2±15	57.8± 2.7	53.6
Total	131.4±1.5	111.4±17.1	163.6± 7.9	130
Homogenate	139 ±1.1	110 ± 1.6	162 ±14.1	128,3

If we compare our data with respect to the separate cell fractions in the studied tissues (Table 1), it is easy to conclude that with lower degree of differentiation and increase in mitotic activity of the cells there is an increase in nucleus-plasma ratio and a decrease in the amounts of mitochondria in the cytoplasm. In this respect, the cells of the mucosa occupy an intermediate position between the liver cells and those of the lymphatic tissues (spleen, thymus). As to the activity of the two DNA depolymerases (Table 2), the cells of liver and mucosa are characterized by very high activity of DNA-ase I which results in deep splitting of DNA (15-20 mg DNA/g tissue per hour) and a comparatively low activity of DNA-ase II (3-4 mg DNA/g tissue per hour). In the spleen and thymus cells the reverse holds true for the DNA depolymerase activity: high activity of DNA-ase II (8-9 mg DNA/g tissue per hour) and very low activity of DNA-ase I (Table 2).

The intracellular localization of DNA-ase in spleen and thymus is different: DNA-ase II is chiefly concentrated in the hyaloplasm, while the DNA-ase I is preferably in the nucleus and mitochondria. In mucosa and liver a difference in localization of the two enzymes is only indicated, and is not so clearly expressed.

The RNA-ase activity in all the organs studied except the thymus is very high, reaching values on the order of 30-60 mg RNA per g of tissue per hour. (Table 2). The greater part of the RNA-ase activity is seen in the cytoplasm, but its distribution between the different fractions of the cytoplasm differs for different organs: in the liver and spleen, the enzyme is concentrated preferably in the mitochondria; for the mucosa the characteristic location of most of the enzyme is in the hyaloplasm.

Human and animal tumors. The data on intracellular localization of proteins shown in Tables 3 and 4 gives some idea of the cell structure of the human and animal tumors which we studied. As the Tables show, from 30-50% of the protein is usually concentrated in the cell nuclei, while the mitochondria contain 10-30%, the microsomes 7-15%, and the hyaloplasm 50-70%. Such a ratio for cell components is usually characteristic of little differentiated, actively dividing cells.

It is important to observe that these figures on the relation of the different structural elements of tumor cells are very approximate, since during fractionation of the tumors we never succeeded in separating fully the nuclei from the elements of connective tissue stroma, and since the chromatin and nucleoplasm of dividing cells during fractionation are distributed between the elements of the cytoplasm.

In Tables 5 and 6 we give the results of determinations of activity of nucleic acid depolymerases in animal and human tumors. We studied the transplantable sarcomas 45 and M-1 and found that in respect to activity of DNA-ase II and DNA-ase I they resembled spleen and thymus, that is, depolymerization of DNA proceeded more actively in the first stage than in the second. However, the absolute value for the activity of both depolymerases was 2-3 times lower than in the normal tissues which indicates the general suppression of the process of DNA depolymerization in the tumors studied. The activity of RNA-ase in these tumors was

TABLE 2

Activity of Nucleic Acid Depolymerases in Homogenate and Cell Fractions of Normal Tissues (decrease of DNA and RNA in mg/g tissue per hour)

Fraction	DNA-ase II	DNA-ase I	RNA-ase
	Liver		
Nucleus	0.44±0.08	2.8 ±0.2	4 9±0.3
Mitochondria	1.88±0.25	10.6 ±0.4	18.4±1.0
Microsomes	0.25±0.08		
Hyaloplasma	0.43±0.20	0.8 ±0.2	8.7±0.5
Total	3.0 ±0.18	14.2 ±0.8	32.0±1.2
Homogenate	3.06±0.19	14.7 ±1.0	31.5±1.7
	Mucosa		
Nucleus	0.43±0.37	3.28±1.16	11.1±1.8
Mitochondria	0.83±0.07	3.37±0.41	11.8±1.2
Microsomes	0.44±0.1	2.37±0.17	7.7±1.2
Hyaloplasma	2.33±0.4	8.15±0.38	22.9±0.98
Total	4.04±0.8	17.17±1.7	53.5±2.8
Homogenate	3.77±0.55	14.4 ±1.48	53.4±1.2
	Spleen		
Nucleus	1.35±0.1	1.18±0.3	18.8±1.46
Mitochondria	2.32±0.41	0.82±0.49	21.9±5.5
Microsomes	0.41±0.29	0.26±0.06	5.2±0.9
Hyaloplasma	4.05±0.89	0.70±0.14	10.2±1.9
Total	8.13±1.1	2.96±0.74	56.1±7.9
Homogenate	8.55±0.3	2.63±0.1	48.8±4.7
	Thymus		
Nucleus	1.39	0.85	3.94
Mitochondria	0.88	0.54	2.53
Centrifugate	3.35	0.46	2.95
Total	5.62	1.85	9.42
Homogenate	6.20	1.98	10.1

TABLE 3

Content and Intracellular Distribution of Proteins in Transplantable Rat Tumors (in mg per 1 g wet weight of tissue)

Fraction	Sarcoma 45	Sarcoma M-1
Nucleus	45.0±4.3	26.6±1.0
Mitochondria	12.2±2.8	24.4±2.4
Microsomes		8.3±1.0
Hyaloplasma	66.3±4.4	53.2±3.1
Total	123.8±4.4	112.5±2.3
Homogenate	111.5±3.9	109 ±3.6

TABLE 4

Content and Intracellular Distribution of Proteins in Human Tumors (in mg per 1 g wet tissue)

Fraction	Angiosarcoma	Sarcoma of soft tissue	Mixed tumor of the testicle	Seminoma
Nucleus	33,5±4,8	25,4± 3,9	26,1±3,0	30,7
Mitochondria	15,9±3,6	21,6± 0,8	17,4±4,0	31,6
Microsomes		16,6± 1,4	15,3±1,6	
Hyaloplasma	14,0±4,6	41,6± 5,8	43,0±4,3	70,5
Total	93,4±1,1	105,2±11,4	101,8±3,0	132,8
Homogenate	93,5±0,6	101,3±10,8	98,0±3,6	121,0

sufficiently high (8-10 mg RNA per g of tissue per hour). As to the Ehrlich ascitic tumor, we were unable to determine the value for the activity of DNA-ases in it with accuracy, since during incubation of the homogenate of the ascitic cells and their fractions with DNA we usually found not depolymerization of the added substrate, but use of the free nucleotides and nucleotides for the synthesis of nucleic acids. We found the same effect in determining the activity of DNA-ases in sarcoma M-1 (Table 5).

TABLE 5

Activity of Nucleic Acid Depolymerases in the Homogenate and Cell Fractions of Transplantable Rat Tumors (decrease in DNA and RNA in mg/g tissue per hour)

Fraction	DNA-ase II	DNA-ase I	RNA-ase
	Sarcoma		
Nucleus	0.47±0.18	0.52±0.1	1.47±0.37
Mitochondria	0.70±0.1	0,59±0.06	0.99±0.19
Centrifugate	1.80±0.4	0.36±0.12	2.54±0.10
Total	2.97±0.5	1.47±0,24	5,0 ±0,43
Homogenate	2.7 ±0.7	1,41±0.2	5.56±0.8
	Sarcoma M-1		
Nucleus	0.93±0,3	0.51±0.1	1,02±0.28
Mitochondria	1.72±0.15	0,46±0.07	3,25±1.02
Microsomes	0.28±0,06	0,16±0,03	2.28±0.25
Hyaloplasma	1,72±0.19	0.03±0.16	1.65±0,88
Total	4.65±0.37	1,10±0.16	8,2 ±1.12
Homogenate	4.2 ±0.51	1,15±0.19	2,98±0,48

In the human tumors which we studied the rate of the DNA depolymerization processes also could not be established with sufficient accuracy, not only because of the slight activity of both DNA-ases, but chiefly because of the simultaneous active process of synthesis of nucleic acids with use of low molecular weight substrates. The activity of RNA-ase in human tumors was very high, from 20 to 40 mg RNA per g of tumor per hour. Evidently, the entire amount of ribonucleotide formed in tumor cells as a result of depolymerization of RNA is quickly used for resynthesis of nucleic acids, since the total amount of free nucleotides in the tumors studied is small.

TABLE 6

Activity of Nucleic Acid Depolymerases in Homogenates and Cell Fractions of Human Tumors (decrease in DNA and RNA in mg/g tissue per hour)

Fraction	DNA-ase II	DNA-ase I	RNA-ase
Angiosarcoma			
Nucleus	0.25	0,63±0,1	7.45± 1.6
Mitochondria	0.23	0,31±0.1	5.39± 1.8
Centrifugate	0 46	0.32±0.2	10.5 ± 3.2
Total	0.94	1 26±0.2	23.3 ± 2,6
Homogenate	0.92	1.7 ±0.57	18.7 ± 0.4
Sarcoma of soft tissue			
Nucleus	0.42±0.11	0 72±0,15	8,4 ± 0.6
Mitochondria	0.36±0.13	0.49±0.1	8.3 ± 3.0
Microsomes	0.03±0.03	0.19±0.08	2.7 ± 0.5
Hyaloplasma	0.82±0.48	0.91±0.24	11.7 ± 3.0
Total	1,63±0.54	2.31±0.23	31.1 ± 6.9
Homogenate	1.5 ±0.1	2.57±0.57	32,9 ± 2.5
Mixed tumor of testicle (teratoma; chorioepithelioma			
Nucleus	0,25±0.14	0.36±0.11	6.7 ± 2,3
Mitochondria	0.11±0.05	0.29±0.04	10.6 ± 3,5
Microsomes	0,10±0.03	0,13±0.07	4.4 ± 1.3
Hyaloplasma	0.37±0.67	0,65±0.4	15,2 ± 6.6
Total	0,09±0.47	1.53±0.32	36.9 ±12.8
Homogenate	1,73±0,52	2.35±0,77	35.5 ±11.7
Seminoma			
Nucleus	0.19	0,72	6.6
Mitochondria	0,24	0.48	5.3
Centrifugate	1,08	1,35	9.1
Total	1,51	2.55	21,0
Homogenate	1.92	4.0	17.6

DISCUSSION OF RESULTS

The experimental material which we have obtained characterizes the absolute rate and relation of the two successive steps in DNA depolymerization in different normal tissues and tumors and permits us to divide the tissues into two groups.

In the first group we place the highly differentiated normal tissues: liver and mucous membrane of the small intestine.

The characteristic sign of this tissue is the high intensity of the second stage of DNA depolymerization (DNA-ase I) with a considerable slowing of the initial part of this process (DNA-ase II).

The biochemical significance of such a disproportion between the two successive steps in DNA depolymerization becomes clear if we consider the processes not in isolation but as the total of interrelated reversible reactions which make up the linked complex process of DNA synthesis. The rate and direction of each part of the process, the details of which are not yet clear, depend on the presence of initial energy, concentration of substrates, enzymes and coenzymes, and on the rate of the preceding and following steps in the chain. Since in the cells of the liver or mucous membrane of the small intestine there is a very active DNA-ase I system, responsible for deep depolymerization of DNA, then actually this system will attack not only the products of

incomplete splitting of DNA by DNA-ase II, but obviously also any intermediates in the synthesis of DNA. Thus, DNA synthesis will be blocked to a greater or less degree in the intermediate stages.

The considerable excess of DNA-ase I over DNA-ase II in highly differentiated normal tissues practically excludes the possibility of renewal of DNA by reutilization of the intermediate products of its splitting, and, probably, the synthesis of DNA in such tissues goes chiefly de novo from low molecular weight precursors. It is true that the high activity of RNA-ase in this group of tissues does not exclude the possibility of use of ribonucleotides as substrates for the synthesis of DNA.

In the second group we consider it possible to combine all the actively proliferating tissues, normal (spleen, thymus) as well as tumors. The most characteristic sign of the tissues of this group is the sharp decrease in rate and depth of depolymerization of DNA as compared to the highly differentiated normal tissues. The difference between the proliferation of normal and cancerous tissues has more a quantitative than a qualitative character. Thus, in spleen and thymus the absolute value for the activity of DNA-ase is 2-3 times higher than in the transplantable tumors of animals, but the ratio between the activity of DNA-ase II and DNA-ase I is shifted in the same way toward DNA-ase II. In the Ehrlich ascitic tumor and in human tumors both stages of DNA depolymerization are sharply inhibited.

All the above forces us to assume that one of the peculiarities in the metabolism of proliferating normal and cancerous cells is their ability to reutilize the intermediate products of DNA depolymerization for its synthesis.

Such a mechanism evidently assures active cell division and great rapidity in renewal of DNA, independent of the presence in the medium of the simpler precursors. On the other hand, the high activity of the RNA-ase assures retaining in the cell a sufficiently high concentration of ribonucleotides as a general substrate for the synthesis of both RNA and DNA.

We can assume that the synthesis of DNA de novo plays only a secondary role in the tissues of this group.

Our theory of the possibility of use by cancer cells of specific high molecular weight precursors of DNA for its resynthesis finds confirmation in the work of Forssberg [9] who observed active uptake of labeled C^{14} by specific DNA or RNA or products of their partial depolymerization in the nucleic acids of Ehrlich ascitic tumor cells.

The data of Davidson [10] on the deficit of enzymes which take part in the synthesis of purines in Ehrlich ascitic tumor cells and bone marrow also favors the hypothesis of the singularity of the parasitic type of biosynthesis of nucleic acids in tumors and actively proliferating normal tissues.

The discovery of more direct experimental confirmation of this hypothesis will be the subject of our further work.

SUMMARY

Determination of the activity of DNA-ase II and DNA-ase I in homogenates of normal and cancerous tissue has led to the detection of a difference between these tissues in respect to the two successive stages in the depolymerization of DNA.

According to the evidence of our investigation, the tissues can be divided into two groups: a) highly differentiated normal tissue (liver, intestinal mucosa); b) actively proliferating normal tissue (spleen, thymus) and tumors. The first group of tissues is characterized by a very intense second stage in DNA depolymerization with considerable delay in the initial step of this reaction. For the second group, the actively proliferating tissues, the characteristic is a sharp decrease in rate and depth of DNA depolymerization compared to the tissues of the first group.

The results permit us to assume the occurrence in actively dividing cells of normal and cancerous tissue of a supplementary mechanism for the biosynthesis of DNA by using the intermediate products of its depolymerization as substrates.

Received March 28, 1958

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THE ULTRAVIOLET FLUORESCENCE OF CRYSTALS OF AROMATIC AMINO ACIDS

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In recent years there have been a number of studies on the fluorescence of solutions of aromatic amino acids [1-4].

The first maximum in the ultraviolet fluorescence spectrum of the amino acids tyrosine and tryptophan was established by us [5] in work in which the results were obtained on powder of the pure substances. In the present work we have studied quantitatively the fluorescent spectra of microcrystals of three aromatic amino acids, tryptophan, tyrosine, and phenylalanine; the tryptophan and tyrosine were used in the form of two optical isomers, L (levorotatory) and DL (mixture of levo- and dextrorotatory).

The experiments were carried out with an ultraviolet microscope fitted with a spectrographic nozzle constructed by one of us [6]. This nozzle permits us to photograph on one photoplate the immediately enlarged image of the preparation with a projection of the spectrograph slit, and the absorption or fluorescence spectrum of the preparation on the line of separation caused by the spectrograph slit. From this spectrogram we can always find the absorption or fluorescence bands which correspond to any part of the micropreparation [7].

The light source was a mercury quartz lamp SVD-120A. Fluorescence was excited in the 275-250 $m\mu$ portion of the spectrum, isolated by a chlorine-bromine gas light filter. In order to decrease the quantity of ultraviolet light which fell on the objective of the microscope in exciting fluorescence in the preparation we placed a dark shade in the plane of the diaphragm aperture of the condenser. For the same purpose, in photographing the fluorescence, a WG-7 light filter (from a Schott set) was placed under the photographic chamber; this strongly reduced the light with wave length less than 290 $m\mu$ and easily passed the longer wave lengths. For photographing the fluorescence we used a highly sensitive Astro-Plattern film (from Agfa), especially for long exposures. The resulting fluorescence spectra were examined on a microphotometer MF-2. At first, using an ultraviolet spectrosensitometer FSR-9, we tested the film used for fluorescence photography and constructed the characteristic curve (relation of density of darkening to logarithm of amount of illumination) for the portion of the spectrum in which the fluoresence was photographed. Using these results and also the effects of the light filter WG-7, we obtained the curve of distribution of energy in the fluorescence spectra of the amino acids which we studied.

In Fig. 1 we give the spectrogram of the crystals of tryptophan, tyrosine, and phenylalanine. Under the photograph of the crystals are the fluorescence spectra of the corresponding amino acids, and at the side of the spectra is the mercury spectrum which serves as a scale of wave length. The photograph of the crystals was obtained in ultraviolet light with wave length 275-250 $m\mu$. The spectral width of the slit was 2 $m\mu$.

Figure 2 gives the curves of energy distribution in the fluorescence spectra of the studied amino acids, obtained by photometry of the spectrograms. In view of the weak absorption by the fine amino acid crystals of light with $\lambda > 290 m\mu$, we assume that reabsorption of fluorescent light by the crystals does not occur. We also assume the absence of selectivity in the passage of the fluorescent light by the optical system of the spectrograph in the spectral region 290-430 $m\mu$.

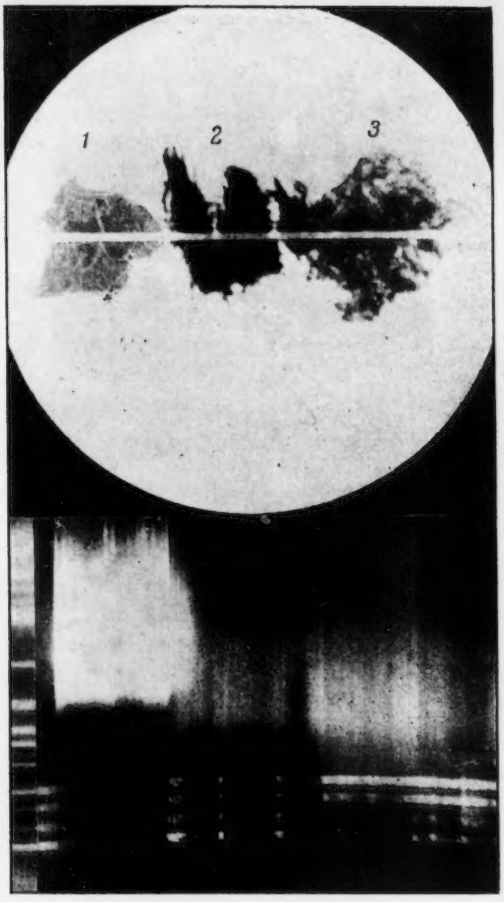

Fig. 1. Spectrogram of microcrystals of aromatic amino acids. 1)Tryptophan; 2) tyrosine; 3) phenylalanine.

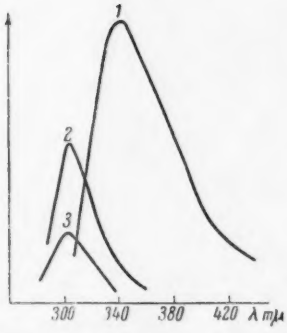

Fig. 2. Curve of energy distribution in the fluorescent spectra of tryptophan, tyrosine, and phenylalanine: 1) DL-tryptophan; 2) tyrosine; 3) phenylalanine. On the ordinate axis is the brightness of fluorescence in relative units.

The positions of maximum radiation for the studied amino acids are the following

Name of Amino Acid	λ_{max} in mμ
DL-tryptophan	345-344
L-tryptophan	336-335

(continued)

DL-tyrosine	303-304
L-tyrosine	303-304
Phenylalanine	301-300

The values for maximum radiation of DL-tryptophan and DL-tyrosine in the solid state agree with the results for the maximum radiation of water solutions of these amino acids given in the work of Teale and Weber [1]; the maximum fluorescence for phenylalanine is shifted compared to that for phenylalanine solutions by 20 $m\mu$ in the long wave field.

Received March 31, 1958

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GLYCOPEPTIDES FROM THE PEPSIN HYDROLYZATES OF EGG ALBUMIN

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Most of the proteins isolated from animal and plant tissues contain some quantity of carbohydrate. The nature of the bond between the protein and the carbohydrate part of the molecule has remained unsettled until now. It is well known that these bonds can differ greatly in stability and chemical character.

In some proteins the carbohydrate is attached very weakly and can be split by repeated reprecipitations or by dialysis (part of the carbohydrate in the protein of blood serum [1]), others require a more strongly acting reagent for this split, one which destroys the hydrogen bond (some of the carbohydrates in microbe proteins are split off by treatment with phenol); among the mucoproteins which contain carbohydrates with acid properties, there are ionic bonds; finally, in a whole series of other proteins the carbohydrates are bound so stably that a covalent chemical bond must be destroyed for their separation. Many serum and tissue proteins belong to this class, among them those of egg albumin. In connection with this variety in the character of the bond there is also variety in biological functions which the carbohydrate–protein complexes carry out in the organism. Many have studied the role of carbohydrates in giving specific functions to different proteins in the protective reactions of the organism. It has been established that the group specificity of blood proteins is assured by the carbohydrate part of the molecule. The same part plays a definite, though still not fully understood role in the origin of the specific antigen properties of the proteins. The carbohydrates play a major part in giving the proteins definite plastic and mechanical properties (in collagen, mucoproteins, mucin); the presence of carbohydrates has also been shown in some hormones, antibiotics, and other biologically active substances (heparin, gonadotropic hormone, etc.).

The difficulty in establishing the manner of attachment of carbohydrates to the protein depends chiefly on the instability of the bond and the absence of methods for the partial destruction of the protein molecule without simultaneously splitting the carbohydrate. Grassmann and co-workers [2] partly hydrolyzed collagan with $Ba(OH)_2$, specifically oxidized the carbohydrate with HIO_4 in one case, and reduced the ether-linked groups by $LiAlH_4$ in another, and showed that the carbohydrate complex probably formed a bridge between the peptide chains of the collagen. From this he assumed that at one end of the chain there was an ester bond, at the other, an O-glucosidal bond. There are short papers on the isolation of carbohydrate-containing products by destruction of protein from casein [3] and fibrin [4]. Finally, in very recent months there have appeared two papers with more concrete data on the isolation of individual glycopeptides. In a letter to the editor Rosevear [5] describes the isolation of a hexapeptide bound to a polysaccharide from γ-globulin hydrolyzed by papain. The point of attachment is, in all probability, the β-COOH group of aspartic acid. After hydrolysis of egg albumin by chymotrypsin, Cunningham and others [6] isolated from the undialyzed protein residue a glycopeptide, in which they found N-terminal tyrosine, aspartic acid, leucine at the C-end, and also threonine, valine, and serine. Thus, chymotrypsin does not split the carbohydrate-peptide bond in egg albumin. On the other hand, from the work of Neuberger it has been shown that prolonged hydrolysis of egg albumin in trypsin fully separates the carbohydrate complex from the protein [7]. However, when we used only a short treatment with trypsin, we succeeded in isolating from egg albumin hydrolyzate several peptides with similar amino acid composition which were bound to carbohydrate complexes [8]. The amino acid composition of these peptides differed considerably from that established for the glycopeptides of Cunningham. Hence, enzymatic splitting is one of the methods for breaking up proteins which can be used for the study of the carbohydrate-protein bond.

In the present paper we describe the study of the pepsin hydrolyzate of egg albumin. As the experimental material shows, the hydrolyzate obtained after short action of pepsin also contains peptides chemically bound to carbohydrates which proves the possibility of using the destruction of egg albumin by pepsin to study the carbohydrate-protein bond.

METHODS

Preparation of the pepsin hydrolyzate of the protein. To a 2% solution of crystalline egg albumin in 0.1 N HCl (pH ~2) after 2-min boiling and cooling was added crystalline pepsin in an amount equal to 1% of the weight of protein. The resulting solution stood for 1 hour at 37° and was then heated to boiling for 5 min to inactivate the enzyme. After 10-day dialysis against water at +5° and evaporation in a vacuum two fractions of hydrolyzate were obtained: A – the dialyzate (20-29% of the taken protein), and B – the high molecular weight part of the hydrolyzate which did not pass through a cellophane membrane.

Quantitative determination of carbohydrate was carried out by the orcinol method of Sorensen [9] on an FEK-M photoelectric colorimeter. Mannose was used as the standard.

Precipitation by phosphotungstic acid was carried out under the conditions given in the work of Neuberger [7], namely: a 20% solution of phosphotungstic acid in 0.1 N HCl was added to the acidified pepsin hydrolyzate until precipitation of protein ceased. After 15 hours in the cold, the residue was separated and carefully washed with 5% phosphotungstic acid. After removal of the acid from the precipitate and from the filtrate, the content of nitrogen and carbohydrate was determined in the latter

Separation by Ion Exchange

Preparation of the carboxyl resin KMG. The ground and sifted resin was washed with water and 1 N HCl, then heated three times for 3 hours with 2 N NaOH, washed free of alkali with hydrochloric acid to an acid reaction, and then free of acid with water to a negative reaction for Cl'. The exchange capacity of the resin was 8.0 m·Eq/g; swelling in water was 500%.

Separation on KMG resin. 50 ml of pepsin hydrolyzate which contained about 50 mg of nitrogen was contrated to 2 ml and passed through a column of KMG (10 × 0.9). The column was washed with water to a negative reaction with ninhydrin. The collected solutions not held back by the cationite were used for further separation on an anionite resin. The column was washed with 0.2 N NH_4OH to remove the basic peptides. Later washings with more concentrated NH_4OH solutions did not extract any more adsorbed peptide.

Preparation of the anionite resin AN-2F. The resin was ground and washed with water to particle size < 0.25 mm, washed with 5 N HCl to a negative reaction for Fe^{+++}, and then alternately three times with 0.5 N NaOH and 1 N HCl. After the last washing, the alkaline anionite was washed with water. The exchange capacity was 3.12 mEq/g; swelling 160%.

Separation of neutral and acid peptides. The concentrated HCl solution of the peptides which were not retained on the cationite was placed in a column with AN-2F (16 × 1). The column was washed with water to a negative ninhydrin reaction, and the adsorbed compounds were removed with N HCl.

Paper Electrophoresis

Electrophoretic separation was carried out in a buffered solution of pyridine-acetic acid-water (100: 4: 900) at pH 6.5 on Leningrad chromatographic paper. On each strip (width 2.5 cm) we placed about 100 μg of peptide (V = 500, duration 10 hours, i at first 6.5 ma finally 7.5 ma).

Paper Chromatography

The separation of the peptides was carried out with triple passage of the solvent (butanol-acetic acid-water (4 : 1 : 5)) through a slow type of chromatographic paper. For detection of peptides, the paper was sprayed with 0.25% ninhydrin solution in butanol or colored by the method of Reindel and Hoppe [10] (chlorination in a chamber with later passage through a solution of o-tolidine in 0.5% acetic acid). Detection of the carbohydrate was carried out by spraying with an alcoholic solution of α-naphthol, orcinol, or resorcinol with addition of

phosphoric acid [11] or coloration by the benzidine method [12] (oxidation by HIO_4, then spraying with a benzidine solution; white spots appear on a blue background).

RESULTS

The experiments showed that the glycopeptides formed during pepsin hydrolysis have a rather high molecular weight, since they pass through a membrane in dialysis only very slowly (Table 1). The greatest percentage of carbohydrate content is found in the last portions of the dialyzate.

TABLE 1

Distribution of Carbohydrates in the Dialysis of the Pepsin Hydrolyzate

Sample	% Carbohydrate in protein
Initial hydrolyzate of egg albumin (1 hour, 37°, pH 2)	2.2
First portion of dialyzate	1.69
Following portion	6.86
Following portion	14.2
Undialyzed residue	3.66

According to Neuberger [7] the free carbohydrate complex remains entirely in solution when the trypsin hydrolyzate of egg albumin is precipitated by phosphotungstic acid. In order to verify the condition, free or bound, in which the free carbohydrate complex from pepsin hydrolysis occurs, we used precipitation by phosphotungstic acid under identical conditions. In the precipitate we found 2/3 of the peptide and almost 60% of the carbohydrate (Table 2). This fact indicates the presence of carbohydrates bound to peptides. It does not exclude the presence of some amount of free polysaccharide in the unprecipitated fraction of the hydrolyzate although it is more probable that it contains glycopeptides which are not precipitated by phosphotungstic acid because of peculiarities of their structure.

TABLE 2

Distribution of Carbohydrates in Precipition of Pepsin Hydrolyzate by Phosphotungstic Acid

Substance studied	Initial hydrolyzate in mg	Precipitated by phosphotungstic acid in mg	
		precipitate	filtrate
Nitrogen	3.4	2.27	0.73
Peptides	21.3	14.2	4.56
Carbohydrates	3.03	1.74	1.18

Later fractionations by ion exchange and by paper electrophoresis and chromatography showed that the hydrolyzate contained not one, but several glycopeptides. In the Plan and in Table 3 we give the course of such fractionations. As can be seen, in ion exchange separation the carbohydrates are divided between all three peptide fractions. Repeated chromatography of the basic peptide fraction (A-2) on cationite KMG scarcely changes its composition (in fraction 8/A-2 there was 9.1% carbohydrate which became 9.8% after repeated passage). The difficulty with this cationite is the strong irreversibility of its adsorption of basic amino acids.

Electrophoretic separation of the resulting fractions confirmed that the basic peptide fraction contained glycopeptides which migrated slowly to the cathode at pH 6.5. In the acid fraction, the carbohydrate-containing zone scarcely moved from the line of application during electrophoresis (Fig. 1). The number of carbohydrate-containing zones in the electrophoregram was small. However, when further paper chromatography was carried out, there was a better separation of fractions and in each of them some carbohydrate-containing zones were observed. Below we give a scheme of paper chromatography for the basic fraction of the peptides (A-2) showing the separated peptides and the carbohydrate zone (Fig. 2) and also a photograph of an analogous chromatogram showing the peptide zones and also their quantitative content of carbohydrates (Fig. 3). The latter were removed from the separate zones and determined quantitatively by the orcinol method.

Plan of Separation of the Pepsin Hydrolyzate of Egg Albumin

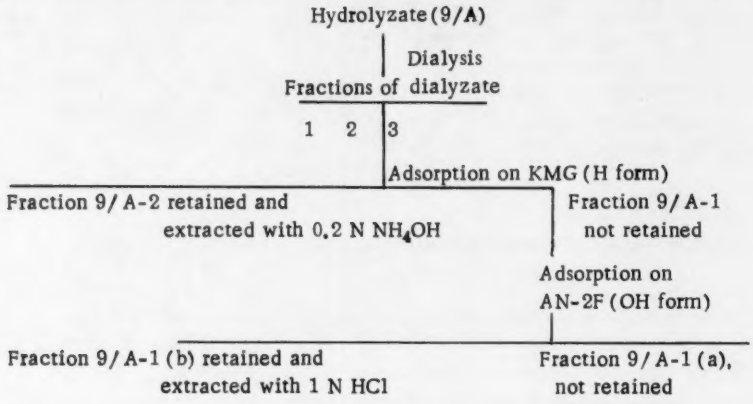
Hydrolyzate (9/A)
Dialysis
Fractions of dialyzate
1 2 3
Adsorption on KMG (H form)
Fraction 9/A-2 retained and extracted with 0.2 N NH4OH
Fraction 9/A-1 not retained
Adsorption on AN-2F (OH form)
Fraction 9/A-1 (b) retained and extracted with 1 N HCl
Fraction 9/A-1 (a), not retained

TABLE 3

Analysis of Peptide Fractions after Chromatographic Separation on Ion Exchange Resins (hydrolyzate 9/A (3): N – 30.28 mg; NH_2 – 3.08 mg; carbohydrate – 11.2 mg)

Substance studied, in mg	Fraction			
	9/A-1	9/A-2	9/A-1 (a)	9/A-1 (б)
Total N	21.66	2.83	8.65	2.34
NH_2	2,22	—	1.25	0.14
Peptides	135.4	17.7	54.0	14.67
Carbohydrates	6.25	2,25	3.25	1.07

Note: For separation on AN-2F we used 80% of fraction 9/A-1

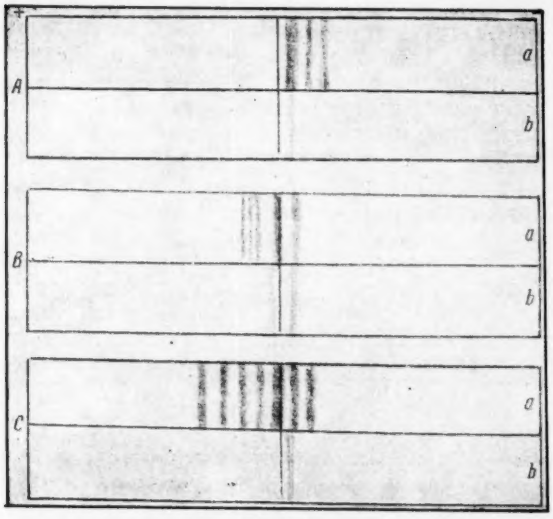

Fig. 1. Electrophoregram of peptide fractions obtained after ion exchange chromatography: A–basic, B–neutral, C–acid peptide fraction. a) Peptide zone, b) carbohydrate zone, colored with α-naphthol.

The quantitative characteristics of the separated carbohydrates fully confirm the qualitative positions of the carbohydrate zone on the chromatogram; part of the carbohydrate moves near the front, but the main mass agrees with the position on the chromatogram of the slowly moving peptide components. Strong washing of the carbohydrate zone for its separation may depend on the presence of several glycopeptides with very similar properties or on some contamination of the glycopeptides by other peptides of the mixture which causes change in R_f.

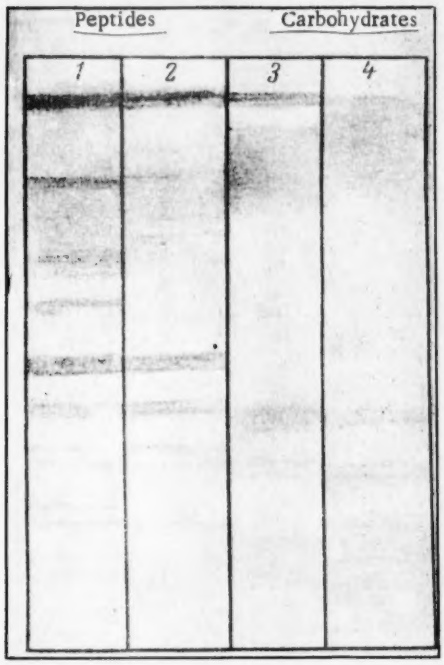

Fig. 2. Triple chromatogram of Fraction 9 / A-2. 1) Colored by tolidine; 2) colored by ninhydrin; 3) colored by α-naphthol; 4) colored by orcinol.

We find similar effects in repeated chromatography of zones removed from the paper by the same solvent. As an illustration, we can describe the following experiment. The pepsin hydrolyzate of egg albumin (11/A) was fractionated by acetone and the last part of the hydrolyzate, which was not precipitated by 98% acetone, and had a carbohydrate content of 14.2%, was taken for study. In paper chromatography of this complex mixture we obtained among other things one wide peptide zone (P-1) which contained traces of carbohydrate, and ahead of it a carbohydrate zone which gave only a weak reaction for peptides with tolidine (C-1) (Fig. 4). Each of these zones was removed by water which was evaporated in a vacuum desiccator, and chromatographed a second time in the same solvent (butanol-acetic acid-water). As Fig. 5 shows, the peptide zone then gives one strong spot at the previous level without signs of carbohydrate, and ahead of it at the level of the carbohydrate zone is a carbohydrate, while behind is a new, purely peptide spot. The carbohydrate zone (C-1) gives three spots at the same levels; of these the strongest is the carbohydrate spot at the previous level, but also there are two new, purely peptide spots formed.

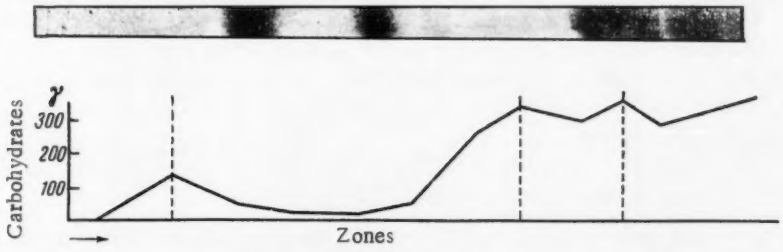

Fig. 3. Triple chromatogram of fraction 10/A-2 showing peptides, and graph of the quantitative content of carbohydrates in the peptide zone.

These facts can be differently interpreted. It seems most probable to us that in the paper chromatography of complex mixtures of high molecular weight peptides there may be reactions between them which lead to changes in R_f. With repeated chromatography of the extracted zones these reactions become less, and the separate components begin to move independently according to their own R_f. In no case did we succeed in isolating from the carbohydrate zone of the pepsin hydrolyzate a fraction which did not contain amino acids.

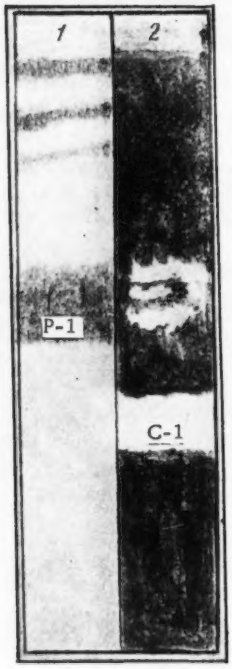

Fig. 4. Scheme of chromatogram of hydrolyzate 11/A: 1) peptides; 2) carbohydrates.

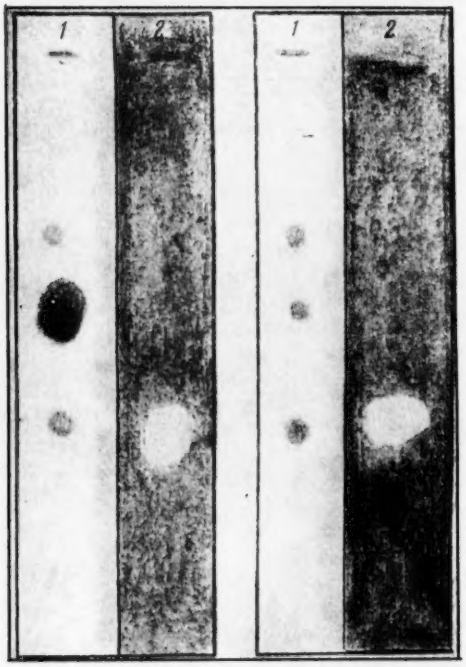

Fig. 5. Scheme of chromatograms of zones P-1 and C-1 from the chromatogram of hydrolyzate 11/A: 1) peptides; 2) carbohydrates.

DISCUSSION OF RESULTS

These experiments lead to the conclusion that the pepsin hydrolyzate of egg albumin contains a large amount of a polysaccharide complex in combination with the peptides. The nature of these glycopeptides differs, since it is possible to separate them into acid, neutral, and basic compounds. Hence their amino acid composition must be different. Evidently, under the action of pepsin the polysaccharide complexes are split with peptide chains of different lengths, which is quite possible in such a short hydrolysis. Actually, we came to the same conclusions about the variety of glycopeptides in the hydrolyzate from trypsin hydrolysis [8], as also did Rosevear [5] who after hydrolysis of γ-globulin by papain isolated three glycopeptides which differed among themselves by one amino acid. At the present time we are studying the amino acid composition and structure of these isolated glycopeptides.

SUMMARY

We have shown that the carbohydrate-protein bond in egg albumin is not split by short hydrolysis with pepsin.

The glycopeptides from the pepsin hydrolyzate have a high molecular weight, since they diffuse very slowly through membranes.

The pepsin hydrolyzate contains several glycopeptides which differ among themselves in physicochemical properties. These glycopeptides can be divided into different fractions by electrophoresis and ion exchange chromatography.

Received May 10, 1958

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REVIEWS

Prof. Dr. Simion Oeriu. Handbook of Biological Chemistry. Bucharest, 1956. (Manual de Chimie Biologica, Bucuresti, 1956).

For a long time the basic textbook on biological chemistry used in the higher educational institutions of Rumania was the course in biochemistry in the French language written by the French professor, Pierre Thomas, who worked for many years in Rumania. Only in 1956 did Prof. Simion Oeriu publish a manual of biological chemistry in the Rumanian language, which is now the basic textbook on this subject in Rumania.

The course which Prof. Oeriu presents in his two volume work includes the important branches of general and medical biochemistry. The first volume presents static (descriptive) biochemistry, discussing biocatalysts and the general laws of the biochemistry of digestion. In the second volume he discusses the present ideas on the chemistry of the metabolism of substances and the metabolism of the separate organs and tissues of the human body.

The descriptive part begins with a detailed consideration of the chemical and physicochemical properties of amino acids and proteins. In regard to this part of the book, we could wish that in preparing a new edition there would be a short section on the methods of electrophoresis and chromatography which now are so widely used in the chemistry and biochemistry of proteins, peptides, and amino acids. We should also mention that another gap is the absence of a description of the results of the excellent work of Sanger in showing the structure of insulin.

After the amino acids and proteins, the author turns to a discussion of the chemistry of sugars and polysaccharides. He then considers the different groups of lipids.

The next section of the book is a detailed discussion of biocatalysts – hormones, vitamins, and enzymes. The last chapter of this division discusses the correlation between the actions of hormones, vitamins, and enzymes. In the last division of the first volume the author considers the biochemical processes which occur in the stomach and intestine during digestion. Thus the author in the first part of the work, increasingly aquaints the reader with the basic ideas of the chemistry of proteins, carbohydrates, and lipids, then turns to consider biocatalysts and their properties, and thus prepares the way for a study of the biochemical laws of metabolism of substances in general and the metabolism of substances in separate organs and tissues.

The second volume of Prof. Oeriu begins with a short survey of methods of investigation of metabolism – the Warburg method, the methods of histo- and cytochemistry, methods of angiostomy and isotopes, and calorimetry. Then come details of carbohydrate, lipid, protein, water, and mineral metabolism. A special division of the course explains the contemporary ideas of the structure, physiocochemical, and biochemical properties of the cell and protoplasm. In the following chapters he explains the biochemistry of the nervous system, the biochemistry of muscle tissue, the biochemistry of bones, the biochemistry of blood. Each chapter of the book is provided with a list of supplementary literature recommended for deeper study of special biochemical questions. These lists contain references to reviews and monographs, and also textbooks. The characteristic of Prof. Oeriu's work is its great attention to work of Russian and Soviet scholars in the field of chemistry in general and biochemistry in particular. We could wish that the author had made a more strict selection of the literature recommended to readers, and had removed from his list some obsolete work.

We have noted some small inaccuracies which should be removed from a new edition of Prof. Oeriu's book. In the historical introduction to the first volume it is incorrect to say that Kirchhoff in 1814 isolated amylase from sprouting grain. As is known, this was done somewhat later by the French scholars Payenne and

Persot. To Kirchhoff belongs the honor of discovering and first describing the effect of enzymatic catalysis. On page 438 the formula for triphosphopyridine nucleotide is not given quite correctly. An error has also occurred in the mention of the discovery of gluten by Beccari (the first communication on this was made in 1728 and was published in the Transactions of the Bologna Academy in 1745). It would be desirable that when the author revises the book he maintains the standard international nomenclature for the stereoisomers of amino acids, sugars, and other compounds.

Evaluating the whole work of Prof. Oeriu, we can feel happy that the students in the higher schools and the specialists in Rumania have a fully modern solid course in biological chemistry written in their national language.

V. L. Kretovich

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Eksptl. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo(po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriia	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Ekspti. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariia	Veterinariia	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniia	Voprosy Pitaniia	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaia Laboratoriia	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiia	Journal of General Biology
Zhur. Vysshei Nerv. Deiatel.	Zhurnal Vysshei Nervnoi Deiatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

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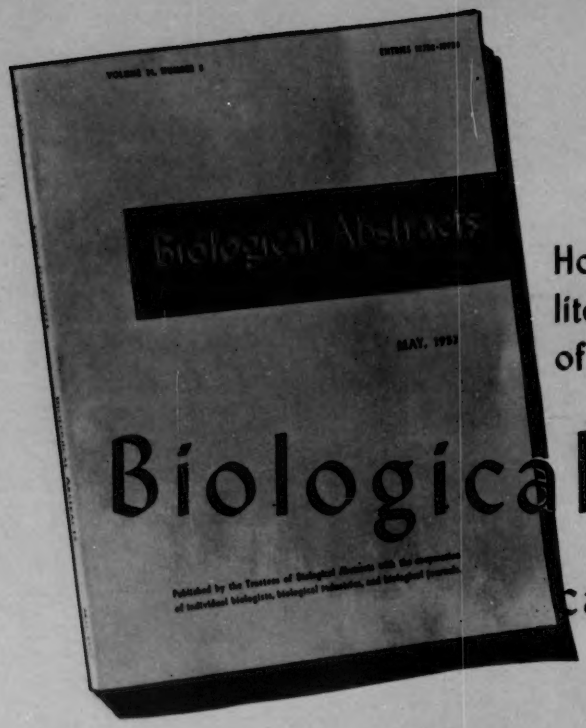

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